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Caseinomacropeptide behaviour in a whey protein fractionation process based on α-lactalbumin precipitation

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This work studied the behaviour of caseinomacropeptide (CMP) in a whey protein fractionation process based on the selective precipitation of α-lactalbumin (α-la) in an acid medium. Three different acids (hydrochloric, citric and lactic) and different operating conditions (protein concentration, temperature and pH) were considered to perform the precipitation step. Under the optimised precipitation conditions obtained for α-la (pH 4, 55 °C, initial α-la concentration around 12 g/l) CMP presents quite similar behaviour to that observed for β-lactoglobulin (β-lg), namely remaining in the supernatant fraction. However, at a lower pH value (3·5) the amount of precipitated CMP increases up to 72% when citric acid is added. This behaviour could be due to the fact that CMP is close to its isoelectric point, which allows a supernatant fraction enriched in β-lg that is almost free from the rest of proteins in sweet whey.

Keywords: Caseinomacropeptide, whey protein fractionation, selective whey protein precipitation.

Whey is the major co-product of cheese-making and casein manufacture in the dairy industry. It consists of milk serum, including low molecular weight solutes such as lactose, milk salts and other minor components, whey proteins and caseinomacropeptide (CMP). Approximately 85% of the total milk used for manufacturing cheese is discarded as whey, still representing an important management problem for medium and small cheese-makers. Cheese whey represents an excellent source of functional proteins and peptides, lipids, vitamins, minerals and lactose.

Whey protein concentrates (WPC) are the most important products obtained in whey processing industries. Commercial WPC may have different protein concentrations (between 35 and 80% on a dry basis) and their uses are widespread, being added to a great number of foods due to their functional properties. However, whey industries must search for new applications of WPC because their utilisation as ingredients in many foods is not enough to absorb total production. Recent years have witnessed an increased interest in separation and fractionation of whey proteins, owing to their nutritional role (Chatterton et al. 2006; Smithers, 2008; Korhonen, 2009; Madureira et al. 2010).

With respect to whey protein fractionation, numerous approaches have been reported using different technologies (ion exchange, selective heat aggregation, chemical additives and membrane technology) but many of them have low efficiency (low protein purity and/or low protein recovery) and others present difficulties at industrial or even pilot plant scale (Lucena et al. 2007). Konrad & Kleinschmidt (2008) described some of these approaches and developed an alternative method to produce pure α-la from whey by means of a combination of membrane and enzymatic treatments (trypsin). α-La obtained in this way was quite pure (93% purity), however, the recovery (15%) was low. An alternative fractionation process is the method based on the selective precipitation of α-la at a pH close to its isoelectric point (pI) under heat treatment (Bramaud et al. 1997a, 1997b; Gezán-Guiziou et al. 1999; Lucena et al. 2006, 2007).

Bramaud et al. (1997b) have shown the key role of calcium in the precipitation reaction of α-la, characterised by a conformational change at acidic pH, around its pI. At this pH (pI 4·2), mild temperature (55 °C) and after adding citric acid (a calcium chelating agent) , together with immunoglobulins (Igs) and bovine serum albumin (BSA), α-la precipitates while most of the β-lactoglobulin (β-lg) remains in solution. There are no conditions under which α-la is the only protein to precipitate. Lucena et al. (2007) studied the precipitation of α-la performed under different conditions in order to optimise the precipitation yield by using lactic acid as a calcium complexant. Gésan-Guiziou et al. (1999) evaluated the purity of the supernatant fraction

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Table 1. Composition of the commercial liquids used as feed

	Sweet whey	WPC-33	WPC-65
pH	6·5	6·8	6·66
Density (g/l)	1023	1050	1060
α-la (g/l)	1·1	5·1	13·4
β-lg (g/l)	4·3	14·8	50·2
BSA (g/l)	0·5	3·8	9·0
Igs (g/l)	0·6	3·8	10·3

obtained in this process, mainly composed of β-lg, and found that the purity is higher using acid whey, which does not contain CMP, than using sweet whey. However, the amount of CMP remaining in solution was not measured.

The total protein content of whey is 5–6 g/l, of which approximately 12% is related to the CMP fraction (Tolkach & Kulozik, 2005). CMP is an acidic, highly soluble and heat stable peptide and is the most abundant peptide in sweet whey besides β-lg and α-la proteins (Thomä-Worringer et al. 2006). The characteristics of this compound as well as its heterogeneity have been widely discussed in the literature (López-Fandiño & Ramos, 1992; Coolbear et al. 1996; Mollé & Léonil, 1995, 2005; Farrell et al. 2004).

During the last few years CMP has been the subject of growing interest due to its beneficial physiological and biological properties. It has a high potential for clinical and nutritional applications and also as a food product structuring agent because of its functional characteristics (Brody, 2000; Casal et al. 2005; Thomä et al. 2006; Kreuß et al. 2008).

One of the main methods used for whey proteins and CMP analysis is high performance liquid chromatography (HPLC). In the previous studies related to the selective precipitation of α-la performed by the authors, CMP behaviour was not taken into account due to the absence of an RP-HPLC method for the simultaneous determination of the CMP and the major whey proteins (α-la , β-lg, BSA, Igs) on a C18 column allowing a correct resolution for all of them. Resmini et al. (1989) used RP-HPLC on a C8 column to quantify whey proteins in raw bulk milk and pasteurised milk. Saito et al. (1991) analysed sweet whey on a C18 column and detected CMP followed by several unresolved peaks corresponding to whey proteins. Similarly, the results reported by Léonil et al. (1997) using a C18 RPC column and Elgar et al. (2000) on a Resource RPC column to separate CMP and whey proteins showed in both cases the aglyco components of CMP were not sufficiently resolved to enable correct peak integration. Thomä et al. (2006) applied a method for the simultaneous separation of CMP on a PLRP-S column in presence of only α-la and β-lg. An improved RP-HPLC method for a rapid and simultaneous analysis of whey proteins and CMP would be of interest. The RP-HPLC method detailed in this work allows simultaneous analysis in a single run using a C18 column.

This work aims to follow CMP behaviour during the selective precipitation of whey proteins in order to obtain a supernatant enriched in β-lg. Pure proteins obtained from whey or WPC could represent an excellent source to obtain valuable peptides via enzymatic hydrolysis. All the experiments presented in this work were performed at a laboratory scale as an initial step before the scaling-up.

Materials and Methods

Feed

Different commercial liquids were used as raw materials to perform the selective precipitation of whey proteins: sweet whey (supplied by Quesería Ovín, Asturias, Spain), and two whey protein concentrates, WPC-65 and WPC-33 (both supplied by Reny Picot, Asturias, Spain). Their compositions are shown in Table 1.

Analytical methods

The analysis of CMP and whey proteins was carried out by HPLC. The HPLC equipment consisted of an Agilent 1200 series chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, auto sampler and a photodiode-array detector. The equipment was controlled by the Agilent ChemStation for LC Systems software which sets analysis parameters, data acquisition and data processing. Separations were performed on a reversed-phase analytical column C18 (Zorbax 300SB-C18, Agilent Technologies) with a silica-based packing (5 µm, 300 Å, 150 mm × 4·6 mm i.d.) at 40 °C and at a flow rate of 1·0 ml min^{-1}.

Ultrapure water (MilliQsystem, Millipore, USA), acetonitrile grade HPLC (Panreac, Spain) and trifluoroacetic acid (TFA) for UV analysis (Panreac, Spain) were used to prepare samples and eluents. All samples were filtered through cellulose acetate filters of 0·45 µm pore diameter (Teknokroma, Spain) and sample injection volume was 20 µl.

A gradient of solvent A containing 0·1% TFA (v/v) in water and solvent B consisting of 0·09% TFA (v/v) in acetonitrile-water (80:20, v/v) was applied. Separations were performed with the following programme: linear gradient from 25 to 48% B in 4 min, followed by an isocratic elution at 48% B for 6 min, then linear gradient from 48 to 55% B in 2 min, from 55 to 65% B in 3 min, from 65 to 90% B in 1 min, held at 90% B for 4 min and then returned to starting conditions in 3 min. Total analysis time per sample was 23 min. Elution was monitored at 214 nm to enable detection of CMP.

Commercially purified bovine whey protein standards (α-la ref.L6010, β-lg ref.L-3908, BSA ref.A3059 and Igs:γ-globulins ref. G-5009) supplied by Sigma (St. Louis, MO, USA) were used to prepare mixed calibration standards. Calibration curves were constructed from different concentration mixed standards in order to estimate the concentration of these whey proteins in the samples. The standard concentrations were weighted to the expected proportional concentrations of each protein in whey. Whey samples were

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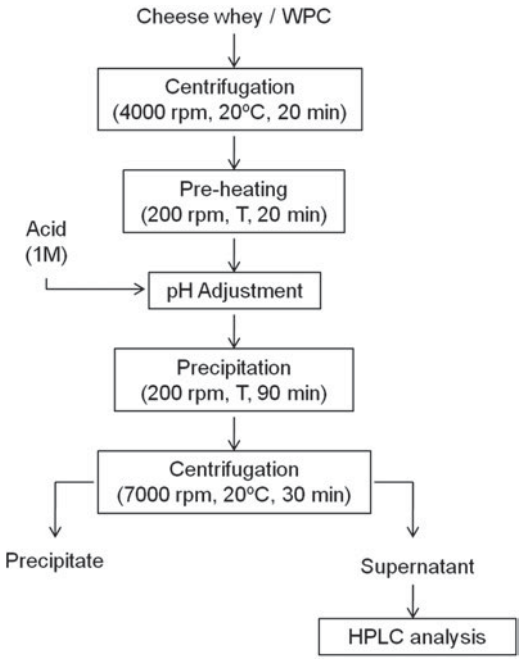

Fig. 1. Flowchart of the experimental procedure.

diluted to obtain protein values within the linear portion of the calibration curve. All the analyses were performed in duplicate and blanks were run prior to and following each sample analysis to minimise carry over. Whey protein concentration was determined from integrated peak areas using the response factors determined with the standards.

Experimental procedure

The precipitation method was similar to that published by Lucena et al. (2006). The raw material was centrifuged (4000 rpm, 20 °C, 20 min) in order to remove suspended solids and fat, and maintained prior to use at 4 °C. All the precipitation experiments were performed using 100 ml of feed and were operated in a batch mode. Temperature and agitation were controlled by means of an incubator (Excella, New Brunswick Scientifics, USA). Samples were preheated at the desired temperature. Then, the pH was adjusted by adding different acid solutions (hydrochloric, citric and lactic acids) at a 1 M concentration. The amount of acid required to achieve the preselected pH value was measured in order to determine the final reaction volume. The acidified feed was maintained under agitation in the incubator at controlled temperature for 90 min. The flask content was then centrifuged at 7000 rpm and 20 °C for 30 min in a discontinuous centrifuge (Kubota 6700, Kubota Corporation, USA). All the experiments were performed in duplicate. The supernatant fraction of this process was analysed by means of HPLC. The flowchart of this procedure is shown in Fig. 1.

The concentration and the amount of proteins in the precipitate fraction were calculated by mass balance, taking into account the amounts of proteins in the initial sample and in the supernatant fraction as well as the final reaction volume. The results were expressed in terms of precipitation yield, which is the percentage of precipitated protein with respect to the initial amount of protein in the feed.

Some parameters that influence the precipitation step were studied. The pH range was between 2·5–6·0, temperature was varied between 30 and 60 °C, and three products with different initial protein content (sweet whey, WPC-33 and WPC-65) were tested.

Results and Discussion

RP-HPLC analysis

In order to identify the CMP peaks in the chromatogram, profiles of sweet and acid whey were obtained. Whey samples were analysed by the method previously described to allow their profiles to be compared. Acid whey is obtained during the making of acid types of cheese and CMP is not released, this being the only protein difference between the two wheys.

RP-HPLC profiles are shown in Fig 2. The CMP compounds eluted in the retention interval between 3·7 and 6·1 min and are absent in the acid-treated sample. The glycosylated fraction eluted between retention time 3·7 and 4·9 min is followed by two well resolved peaks at 5·2 min and 5·9 min corresponding to the aglyco A and B components, respectively. The peak areas of the aglyco fractions were measured to determine CMP behaviour during the selective precipitation.

The RP-HPLC method detailed in this research work allows the simultaneous determination of the major whey proteins and CMP in a single run using a C18 column. The chromatographic profile showed that the individual proteins were baseline resolved from each other enabling correct integration. The order of elution of the major whey proteins resembled that obtained by Resmini et al. (1989) using a C8 RP column and the resolution of CMP species was similar to that previously observed on a C18 RP column (Coolbear et al. 1996). The elution profile of the whey proteins and CMP is related to their average hydrophobicity, with the glyco-CMP fractions (gCMP) eluting earlier than the aglyco-CMP (aCMP) followed by α-la, BSA, β-lg B and A and Igs. This elution order is in agreement with the results reported by Elgar et al. (2000) on a Resource RPC column but, in contrast to these results, the resolution is remarkably enhanced with respect to the separation of the aCMP and gCMP components. The CMP was eluted as an ill-defined series of peaks and the two major peaks, corresponding to the aCMP components of the two principal genetic variants (A and B), eluted after the less well resolved peaks of the gCMP components. The baseline resolved aCMP peaks enabled straight forward integration and the content of CMP in a

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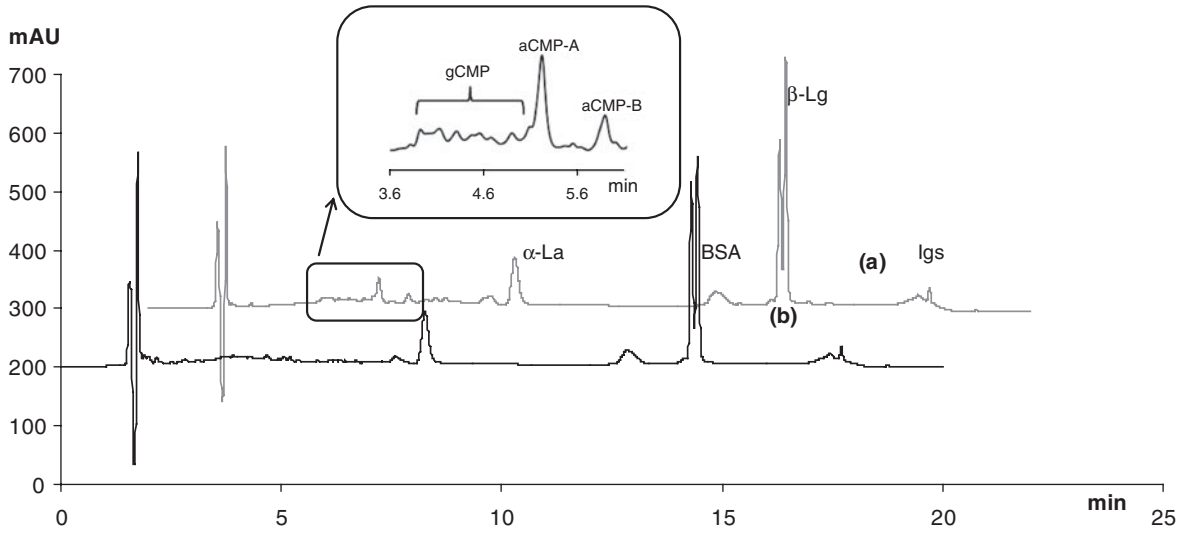

Fig. 2. RP-HPLC chromatogram comparison of sweet (a) and acid (b) whey. Retention times (t_R) of CMP and the major whey proteins are: gCMP, $t_R = 3{\cdot}7 - 4{\cdot}9$ min; aCMP-A, $t_R = 5{\cdot}2$ min; aCMP-B, $t_R = 5{\cdot}9$ min; α-la, $t_R = 8{\cdot}3$ min; BSA, $t_R = 13$ min; β-lg, $t_R = 14{\cdot}4$ min; Igs, $t_R = 17{\cdot}5$ min. Conditions for RP-HPLC analysis as described in the text.

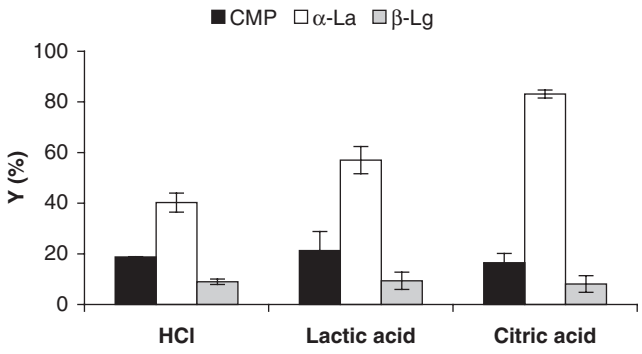

Fig. 3. Precipitation yield (*Y*%) of CMP, α-la and β-lg when three different acids (hydrochloric, citric and lactic) were added. Precipitation conditions: pH 4·0, 200 rpm, 55 °C, 90 min.

sample to be expressed as percentage peak area with respect to the raw material.

Effect of the precipitation agent in the CMP behaviour

To check the effect of adding a calcium chelating agent on CMP solubility, three different acid solutions were added: a mineral acid (hydrochloric acid) and two organic acids which have different complexant capacity (citric and lactic acids). All experiments were performed at the optimum conditions to achieve quantitative precipitation of α-la, according to Lucena et al. (2007). The results are shown in Fig. 3. The Y-axis represents the precipitation yield (*Y*%) of CMP, α-la and β-lg, calculated as the relation between the amount of each protein in the raw material and in the precipitate fraction. Protein precipitation when adding HCl is due to the pH conditions, next to α-la isoelectric point, and must be considered as irreversible precipitation (Lucena et al. 2007). The addition of a calcium chelating agent, such as citric or lactic acid, enhances the precipitation of α-la. Citric acid causes higher α-la precipitation than lactic acid, however use of lactic acid is of interest due to the fact that this chemical can be obtained from whey. CMP exhibited similar behaviour in all the experiments. In all cases an amount of CMP slightly lower than 20% is obtained in the precipitate fraction, which means that its precipitation is not influenced by the type of acid added. In fact, that part of the CMP is entrapped in the sediment as happens with β-lg. To test this point, the precipitates obtained using citric and lactic acids were washed with deionised water adjusting the pH value to 4·0. In both cases up to 40% of CMP and β-lg are removed from the precipitate. The results demonstrate that CMP and β-lg are not precipitated but rather are entrapped in the precipitate.

Effect of the initial protein concentration on the CMP behaviour

In order to find the optimum initial protein concentration and its influence on CMP behaviour several solutions were used as raw materials: sweet whey (1·1 g α-la/l), WPC-33 (5·1 g α-la/l) and WPC-65 (13·4 g α-la/l). Acid solutions of 1 M-concentration were added to the raw materials to reach a final pH value of 4·0. All the experiments were performed at the same operating conditions (pH 4·0, 200 rpm, 55 °C, 90 min). Figure 5 represents the precipitation yield (*Y*%) of CMP, α-la and β-lg when varying the protein concentration of the raw material. According to previous works (Pearce, 1983; Lucena et al. 2007) the initial protein concentration affects the precipitation yield and α-la has a higher tendency to aggregate at higher initial concentration. Concentrated whey gives better results in terms of precipitate removal and

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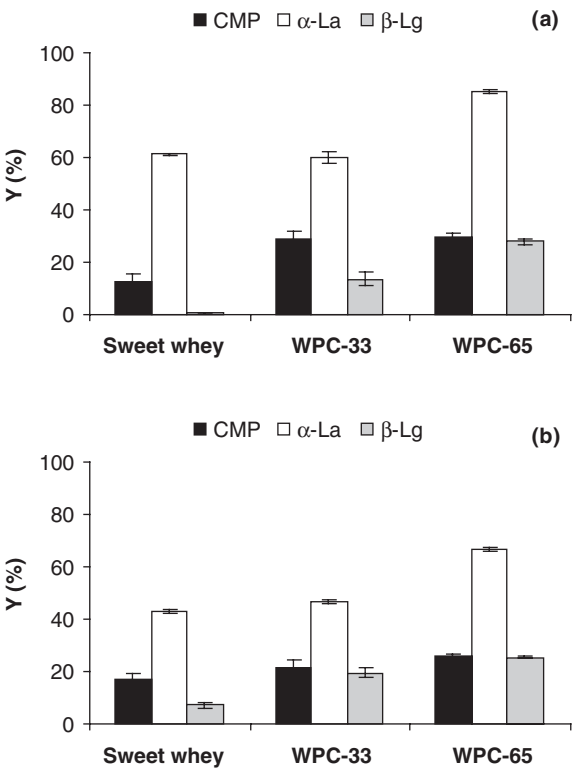

Fig. 4. Effect of initial protein concentration (sweet whey, WPC-33 and WPC-65) on the precipitation yield (*Y*%) of CMP, α-la and β-lg when citric (a) and lactic (b) acids were added: pH 4·0, 200 rpm, 55 °C, 90 min.

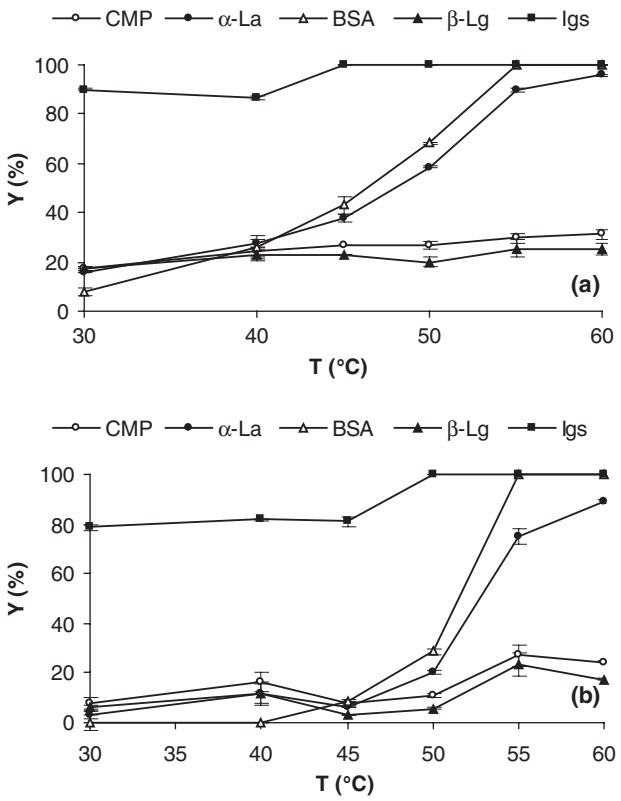

Fig. 5. Effect of temperature on the precipitation yield (*Y*%) of the major whey proteins when citric (a) and lactic (b) acids were used: pH 4·0, 200 rpm, 90 min.

protein recovery per litre of feed. On the other hand, even if CMP and β-lg do not precipitate, the higher the amount of initial protein, the greater the proportion of CMP and β-lg trapped in the sediment. This results in a less efficient separation of the proteins present in the precipitate and supernatant fractions. According to Fig. 4 this effect is less important in sweet whey because the protein concentration is lower than in the case of WPC-33 or WPC-65. The precipitate obtained using WPC 33 and 65 is pastier and more viscous than using sweet whey, probably due to mixing problems. When the precipitation agent used is citric acid in an initial α-la concentration of 1·1 g/l, 10% of the CMP is trapped in the sediment, while this proportion increases up to 29% when the initial concentration is 13·4 g/l.

Effect of the temperature on the CMP behaviour

In Fig. 5, the precipitation yield (*Y*%) of CMP, α-la, BSA, β-lg and Igs is plotted versus temperature. In agreement with the studies published by Lucena et al. (2007), a good precipitation yield was observed at temperatures higher than 55 °C for α-la, BSA and Igs while under these conditions β-lg remains in solution. BSA shows similar behaviour to α-la with respect to Ca^{2+} whereas Igs are denatured, even at low temperatures, due to the low-pH-induced changes in the secondary structure of this protein (Gumpen et al. 1979; Arnoldus et al. 2000). CMP behaviour is similar to that shown by β-lg. The precipitation yield of CMP increased slightly with temperature but did not exceed 27% when using lactic acid. The fact that CMP has high thermal stability compared with other whey proteins accounts for these results (Martín-Diana et al. 2002).

Increasing temperature barely affects the precipitation yield of CMP. However, the precipitation of α-la, BSA and Igs is strongly influenced by this factor, presenting quantitative precipitation yield at temperatures higher than 55 °C in case of α-la. According to these results a precipitation step at pH 4·0 and temperature above 55 °C could allow to obtain a supernatant fraction mainly composed of β-lg and CMP, being the amount of α-la present in this fraction very low.

Effect of the pH on the CMP behaviour

In Fig. 6 (a) and (b) the precipitation yields of CMP and α-la at different pH values are shown. In the case of α-la the precipitation could be considered quantitative for both organic acids at pH between 3·5 and 4·0. According to previous researchers (Bramaud et al. 1997b) the presence of an organic acid with ability to complex Ca^{2+} turns the protein into *apo*- α-la adopting a "molten globule-like" state

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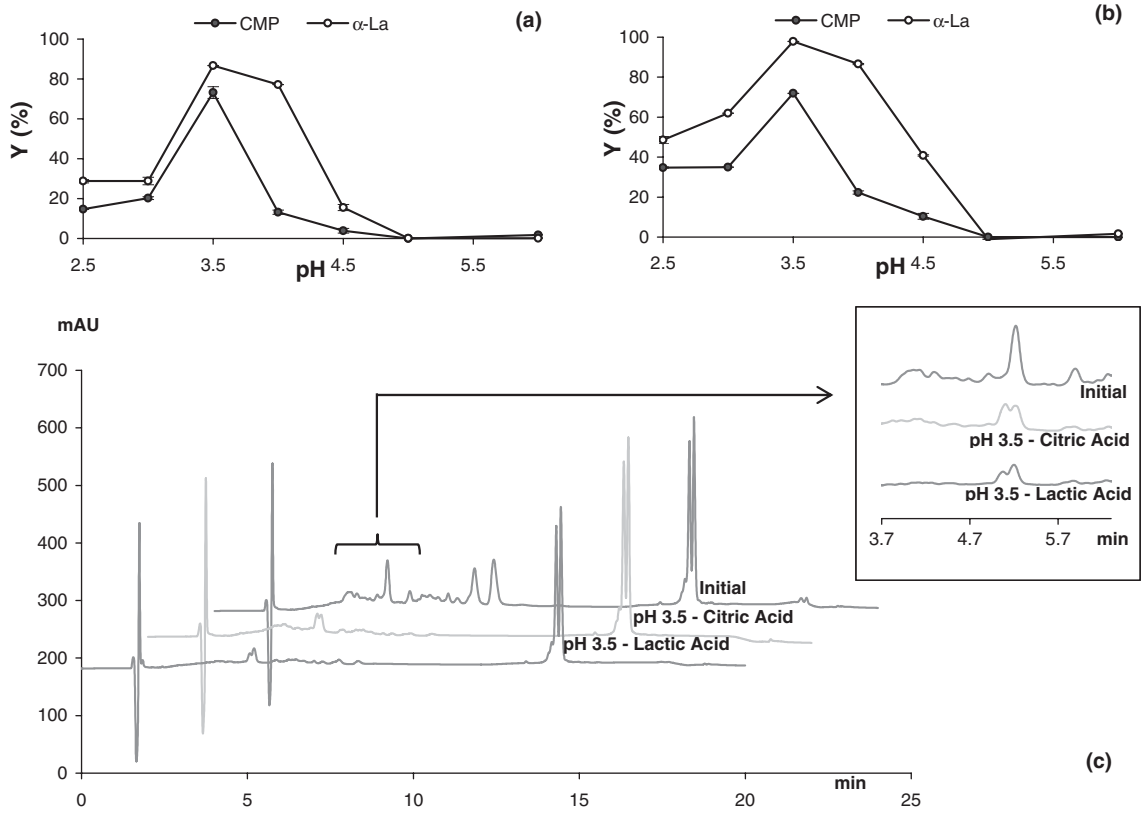

Fig. 6. pH effect on the precipitation yield (*Y*%) of CMP and α-La adding citric (a) and lactic (b) acids. Precipitation conditions: 55 °C, 200 rpm, 90 min. (c) RP-HPLC profile obtained at different pH values adding citric and lactic acids. RP-HPLC analysis conditions as described in the text. CMP behaviour is highlighted.

which is less soluble (Permyakov & Berliner, 2000), and then precipitates. This process seems to be reversible in such a way that when the initial conditions are recovered the protein becomes soluble again, at least partially (Bramaud et al. 1997a; Lucena et al. 2007). CMP behaviour is slightly different from that observed for the rest of the proteins. While α-la, BSA and Igs precipitate at a pH value close to 4·0 and β-lg remains in solution, 70% of the CMP precipitates at a pH value of 3·5. Figure 6 (c) shows the chromatograms obtained when the precipitation is carried out at pH 3·5 adding citric and lactic acids. Of relevance is an important decrease of the peaks representing CMP and the total precipitation of α-la, BSA and Igs. This effect of pH on the precipitation of CMP could be related to the pI of this compound.

As mentioned in the introduction section, CMP is a highly diverse, heterogenic group of molecules due to its genetic variants as well as post-translational modifications. The exact pI of this compound may vary depending on its sialic acid content and the degree of phosphorylation. Nakano & Ozimek (2000), based on the results obtained by anion-exchange chromatography, suggested that all sialyzed gCMP has an apparent pI lower than 3·8. Lieske et al. (2004), using chromatographic analysis carried out with a cation exchange column, proposed a pI value between 3·50 and 3·75 for the entire CMP. Furthermore, according to ExPaSy Molecular Biology Server the pI of total CMP is reported to extend between 3·0 and 4·1. All these studies support the hypothesis that CMP precipitation observed at pH 3·5 could be due to reaching a pH value close to its pI.

Results shown in Fig. 6 have been calculated based on the assumption that entire CMP, both glyco and aglyco fractions, exhibit the same behaviour versus pH. However, as could be noted in the expanded chromatogram represented in Fig. 6 (c) only part of the aCMP-A fraction remains in the supernatant of the precipitation process when the experiment is carried out at pH 3·5 while all aCMP-B and gCMP precipitate. As mentioned in Kreuβ et al. (2009), glyco and aglyco fractions of CMP have different pI values, being these 3·15 and 4·15 respectively. The fact that whole gCMP precipitates when the pH of the experiment is adjusted to 3·5 may be due to its closer proximity to the pI.

Taking into account that CMP represents about 12% of the total protein content of whey and the amount of each protein present in the supernatant fractions, an increase in purity of 10% in β-lg (from 84 to 94%) is observed when the precipitation is carried out at pH 3·5 instead of at pH 4.

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