ELSEVIER

Journal of Chromatography B, 711 (1998) 91–96

JOURNAL OF CHROMATOGRAPHY B

Short communication

Aqueous two-phase systems: a novel approach for the separation of proteose peptones

F.H. Isgrove[a,*], R.J.H. Williams[a], G.W. Niven[b], A.T. Andrews[a]

[a]*Food Chemistry Group, School of Food and Consumer Science, University of Wales Institute, Cardiff, Colchester Avenue, Cardiff CF3 7XR, UK*

[b]*Biotechnology and Enzymology Department, Institute of Food Research, Reading Laboratory, Earley Gate, Whitknights Road, Reading RG6 2EF, UK*

Abstract

Poly(ethylene glycol) and dextran aqueous two-phase systems (ATPS) were developed to facilitate the separation of components of the proteose peptone fraction of bovine milk, which are mostly large casein derived peptides or glycoproteins. These have proved difficult to purify using conventional chromatographic procedures. ATPS exploit differences in hydrophobicity, size and ionic properties of the proteose peptones with a view to developing methods for future large scale preparations of the individual components of this whey protein fraction.

Keywords: Aqueous two-phase systems; Proteose peptones; β-Casein

1. Introduction

The proteose peptone fraction of milk consists of a large number of components including a number of minor proteins and glycoproteins, and many peptides produced in vivo from caseins, mainly by the activity of the indigenous proteinase plasmin [1]. These peptides, representing ~10% of the whey protein fraction, can be collected from skimmed milk using trichloroacetic acid (TCA) precipitation following the heat treatment of milk at 95°C for 30 min, and the adjustment to pH 4.6 in order to precipitate the caseins and heat-denatured whey proteins [1,2]. Investigative studies on the proteose peptones have concentrated on the isolation and characterisation of the four main groups, β-CN-5P (M_r 12 300), β-CN-4P (M_r 3469), β-CN-1P (M_r 9000) and HFPP (hydrophobic fraction of proteose peptones), previously known as PP5, PP8f, PP8s and PP3, respectively [3]. β-CN-5P represents amino acids 1–105 and 1–107 of β-casein and β-CN-4P, 1–28 of the same molecule [1,2] (see Fig. 1). Both components are multiple phosphoseryl containing peptides, and contain the marker sequence SerP–SerP–SerP–Glu–Glu which has been implicated both in anticariogenic activity and the regulation of biomineralisation, protein structure and enzyme activity [4]. A proposed mechanism for the activity of this sequence is based on its ability to localise amorphous calcium phosphate in dental plaque, which by acting as a buffer of free calcium phosphate ions would prevent the demineralisation of tooth enamel by acid from plaque-forming bacteria [4–6]. This has led to commercial interest in the production and isolation of these phospho peptides on an industrial scale [5]. Reynolds et al. [4] write, ‘‘To facilitate studies on the identification, structure, and properties of these peptides, specific methods for their purification and

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00086-3

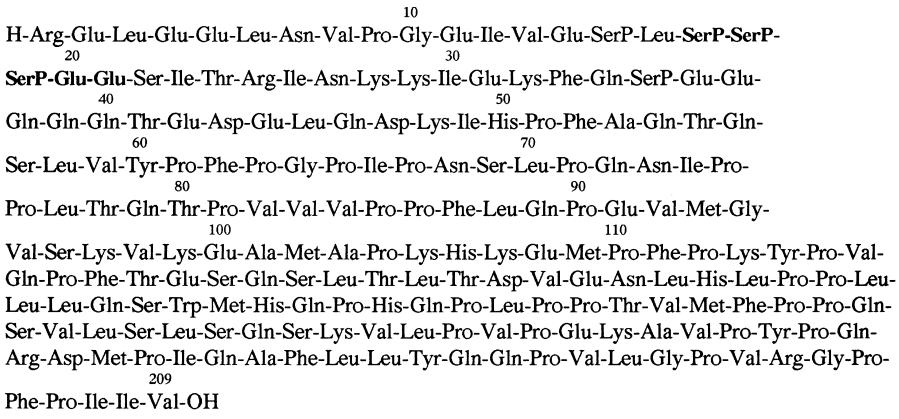

Fig. 1. The amino acid sequence of β-casein. The marker sequence is shown in bold. β-CN-5P consists of amino acids 1–105 or 1–107. β-CN-4P consists of amino acids 1–28.

characterisation are required''. We aim to show how aqueous two-phase systems (ATPS) may be developed as a novel methodology for fractionating individual proteose peptone components on a relatively large scale. We also propose an alternative means of generating peptides of interest by digestion of purified β-casein with plasmin, the resulting fractions of which will be subjected to ATPS for selective purification.

2. Experimental

2.1. Chemicals

Dextran T500 (M_r~500 000), was purchased from Sigma (Poole, UK). Poly(ethylene glycol)s (PEGs) with approximate molecular mass of 1000, 1450, 3350, 8000 and 10 000 were purchased from Sigma. All other chemicals were of analytical grade and purchased from Sigma.

2.2. Proteins and peptides

The total proteose peptone (TPP) fraction was prepared according to Andrews and Alichanidis [1]. β-Casein was prepared according to Andrews and Alichanidis [1] by ion-exchange chromatography (IEC) of total casein on DEAE–cellulose (Whatman DE52). The pH was 7.0, and the buffer contained 3.3 *M* urea. The β-PP fraction was prepared by adding 15 ml of plasmin solution (0.1 mg/ml in deionised water) to 750 ml of β-casein solution adjusted to pH 7.03, containing 45.9 g of β-casein. This mixture was incubated for 120 min at 37°C. Residual β- and γ-caseins were precipitated out by adjusting the pH to 4.6. The precipitate was pelleted by centrifugation (4000 *g*, 15 min). The pellet was washed once with deionised water, centrifuged as above, and the resulting supernatants were pooled. TCA was added to a concentration of 10%, the precipitate was collected on filter paper, washed twice with acetone and air-dried.

2.3. Preparation of phase systems

All ATPS (0.9 ml) were prepared from stock solutions of the polymers (w/w) in deionised water. The total proteose peptone sample was added at a final system concentration of 1 mg/ml. Salt (sodium chloride) was added where listed at final concentrations of 1%, 5% and 10%. The pH was maintained in the systems with 0.1 *M* buffer. The systems were Vortex mixed for 30 s using a Jencons Miximatic, and were left to separate at ambient temperature for at least 1 h. Samples (25 μl) from each phase were extracted using Gilson Microman Positive Displacement Pipettes, diluted with 50 μl of sample buffer containing 5% sucrose and 0.005% bromophenol blue, and added to a polyacrylamide gel electrophoresis (PAGE) gel (25 μl sample applied).

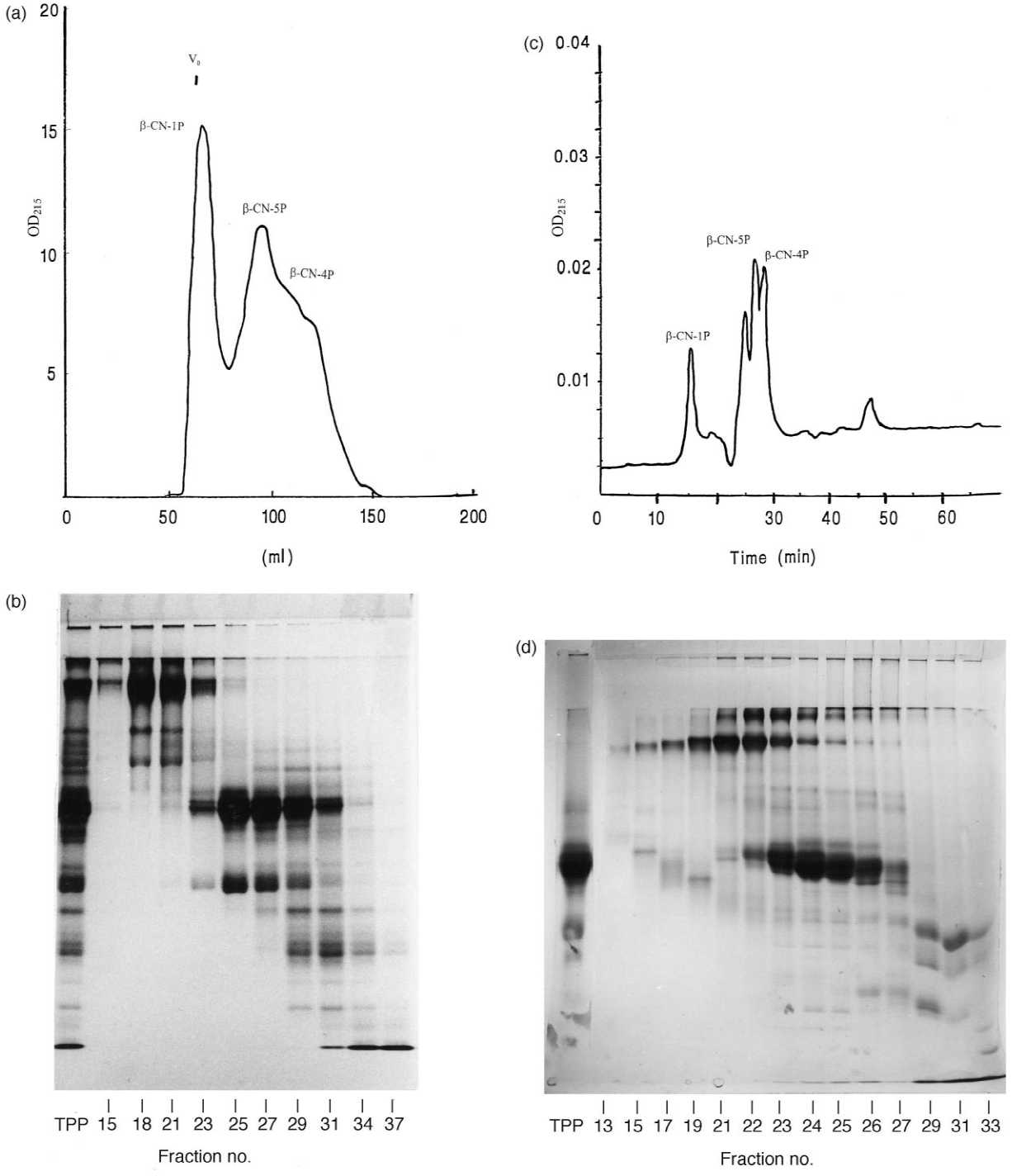

Fig. 2. Conventional methods of fractionating proteose–peptone components. (a) Gel permeation on Sephadex G-75 in 0.1 *M* Na borate buffer pH 7.5, UV absorbance monitoring at 215 nm. (b) Corresponding fractions monitored by PAGE, (TPP control). (c) Gel permeation by HPLC on a Superose 12 HR 10/30 column in 0.05 *M* Tris–HCl pH 7.0 containing 0.25 *M* NaCl, monitoring at 215 nm. (d) PAGE analysis of fractions obtained by ion-exchange chromatography on DEAE–cellulose with 0–0.5 *M* NaCl elution gradient in 0.05 *M* Tris–HCl pH 7.0 buffer.

2.4. Gel electrophoresis

PAGE gels (T=12.5%, C=4%) were prepared according to the manufacturer's instructions (Hoefer Scientific Instruments, San Fransico, CA, USA) and run according to Andrews and Alichanidis [1]. The gels were run at a constant voltage of 280 mV on a Biometra P30 powerpack.

2.5. IEC, gel permeation and high-performance liquid chromatography (HPLC) techniques

Preparative gel permeation was performed on a G-75 Amicon Wright column (550×22 mm) (Stonehouse, UK) the details of which are shown in the Figure legends. HPLC gel permeation analysis was performed using a Dionex DX500 HPLC chromatography system, with a Superose 12 HR 10/30 column (Pharmacia Biotech, St. Albans, UK). A Whatman DE-52 DEAE–cellulose column (110×33 mm) (Maidstone, UK) was used for the ion-exchange data (Fig. 2d).

3. Results and discussion

The gel permeation chromatographic profile in Fig. 2a and the corresponding PAGE photograph in

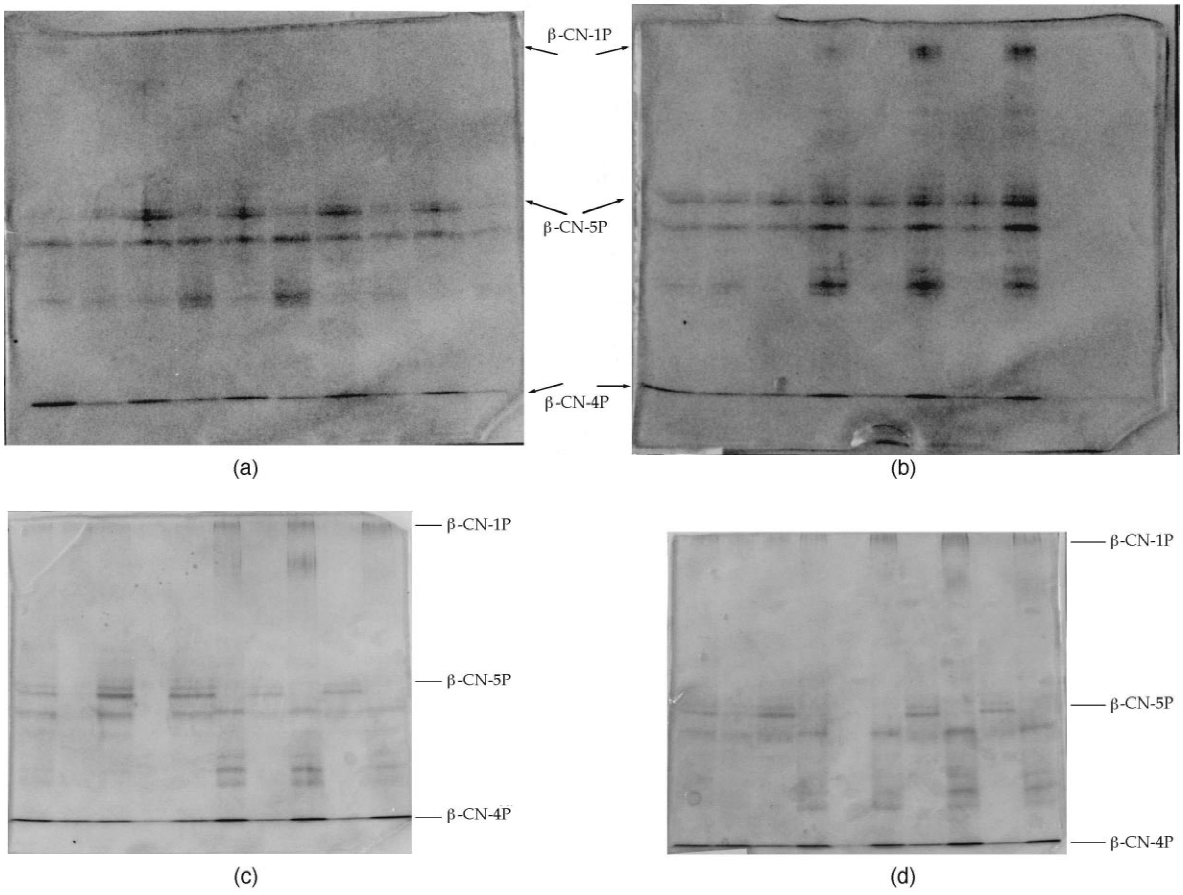

Fig. 3. PAGE gels showing a TPP fraction separated using ATPS. All systems are at pH 7, 0.9 ml in volume, and use starting concentrations of 12% (w/w) PEG and 8% (w/w) Dextran T500 (Dx). Lanes 1 and 2 represent PEG 1000 systems, lanes 2 and 3 PEG 1500 systems, lanes 5 and 6 PEG 4000 systems, lanes 7 and 8 PEG 8000 systems and lanes 9 and 10 PEG 10 000 systems. Odd numbered lanes=PEG phase, even numbered=corresponding Dx phase. (a) Contains 0% NaCl, (b) 1% NaCl, (c) 5% NaCl and (d) 10% NaCl. TPP components were observed to precipitate at the interface between phases, particularly in the lower-molecular-mass systems.

Fig. 2b show that the resolution is not adequate to purify individual components with substantial overlap between peptide zones. Increasing the resolving potential of gel permeation by introducing HPLC methods is also insufficient (Fig. 2c). Ion-exchange separation on a DEAE column likewise shows substantial overlap between peptide peaks, which again indicates inadequate resolution (Fig. 2d).

In view of the inadequacy of conventional purification methods, such as gel permeation and IEC, new methods are required, especially as the best of existing methods (gel permeation) is not suitable for large scale applications. Methods relying on affinity interactions have not yet been investigated. Probably the most promising approach would be to raise antibodies and use immobilised antibody affinity chromatography or affinity precipitation. However, these methods would be both prohibitively expensive on a large scale and unlikely to give good results since most of the components of the proteose peptone fraction are derived from the breakdown of just four related precursor proteins hence causing substantial immunological cross-reactivity. Because of the known advantages of ATPS in terms of scale-up and costs we are therefore exploring its potential.

Fig. 3 shows the effects of PEG molecular mass and sodium chloride concentration on the partitioning of the components of a total proteose peptone fraction. β-CN-5P can be seen to be more selectively extracted into the upper PEG phase with higher molecular mass PEGs. The presence of sodium chloride appears to be more influential on partitioning that the precise concentration used. Systems with a pH of 7 were found to be more effective at selectively partitioning β-CN-5P into the PEG phase, when compared with identical systems at pH 3, 5 and 9 (data not shown).

While PAGE gels provide a good qualitative picture of the phase distribution of the proteose peptone components, we are currently exploring HPLC methods of analysis in order to obtain quantitative results.

The other major objective of this project was to study the formation of proteose peptone components in order to increase and optimise their production. Amounts of the proteose peptone fraction isolated from bovine milk are relatively small, but those components resulting from the natural casein hydrolysis by plasmin could also be generated in larger quantities by suitable in vitro hydrolysis. In order to simplify interpretation, individual purified caseins can be used as the starting material and plasmin treatment of β-casein, for example, would produce

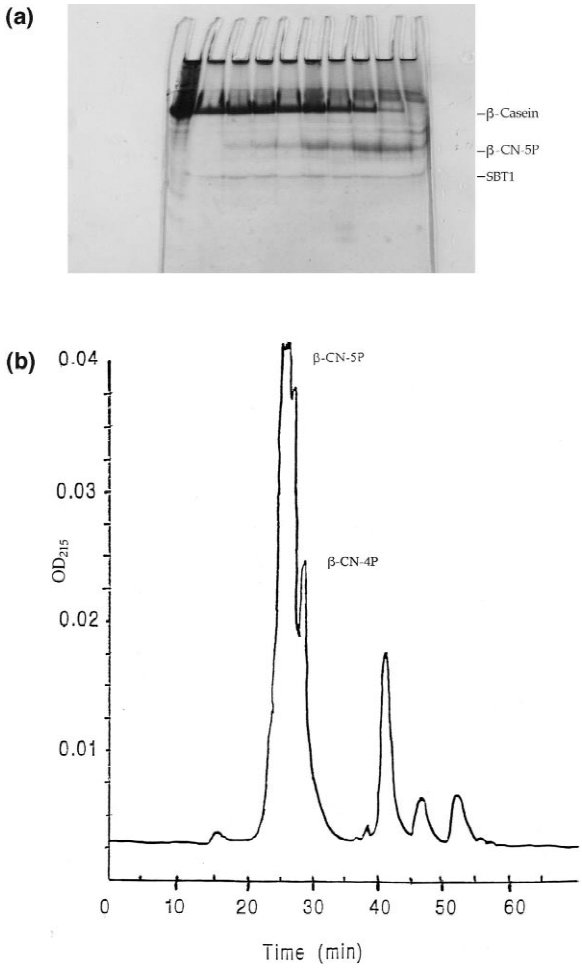

Fig. 4. (a) PAGE gel showing the progressive breakdown of β-casein. Plasmin was added to a solution of β-casein at a ratio of 2 μg plasmin/60 mg β-casein. The reaction took place at 37°C, and was arrested by the addition of 0.1 mg/ml SBTI. Lane 1 shows β-casein prior to digestion. Lane 2 shows β-casein and SBTI. Lanes 3–8 represent the digestion of β-casein following 2, 5, 10, 20, 30, 60, 120 and 240 min incubation with plasmin, respectively. The gel was heavily overloaded with β-casein in order to reveal peptide breakdown more clearly. (b) HPLC profile of peptides produced from β-casein by plasmin digestion. Column used was Superose 12 HR 10/30 in 0.05 *M* Tris–HCl pH 7.0, containing 0.25 *M* NaCl.

both β-CN-5P and β-CN-4P. Fig. 4 shows their formation from β-casein. β-CN-5P is not an end-product of this hydrolysis and is broken down further to β-CN-4P and a number of other peptides, requiring careful selection of suitable conditions. To date, yields have been disappointingly low, (approx. 2 g from 45 g of β-casein), but clearly the bonds at 28–29 and 105–106/107–108 do represent favoured cleavage sites in β-casein by plasmin (Fig. 1).

4. Conclusions

Any commercial exploitation of components of milk proteose peptone is going to require cost-effective large scale fractionation techniques. Gel permeation is not suitable for the required scale, and ion-exchange methods probably have inadequate resolution. Although at a preliminary stage, our results have shown that ATPS appear to offer a useful alternative, and in the presence of NaCl at pH 7.0, there is considerable enrichment of β-CN-5P in the PEG phase. In future work we will seek to optimise this partitioning of β-CN-5P and devise appropriate conditions for the ATPS purification of β-CN-4P and other ''cluster peptides''. Production of these materials from casein precursor molecules will also be studied, as will their recovery from phase-forming polymer solutions.

References

[1] A.T. Andrews, E. Alichanidis, J. Dairy Res. 50 (1983) 275–290.
[2] J.-M. Girardet, G. Linden, J. Dairy Res. 63 (1996) 333–350.
[3] J.-M. Girardet, A. Mati, T. Sanago, L. Etienne, G. Linden, J. Dairy Res. 58 (1991) 85–98.
[4] E.C. Reynolds, P.F. Riley, N.J. Adamson, Anal. Biochem. 217 (1994) 277–284.
[5] N.J. Adamson, E.C. Reynolds, Biotechnol. Bioeng. 45 (1995) 196–204.
[6] N.J. Adamson, E.C. Reynolds, Electrophoresis 16 (1996) 525–528.