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Fractionation of cheese nitrogen using chitosan

Manuela Fernández* & Patrick F. Fox

Department of Food Chemistry, University College, Cork, Ireland

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The use of chitosan (0.02%) to fractionate the water-soluble (WSE) and water-insoluble (WISF) fractions of cheese was studied. Chitosan, a polymer of *N*-acetylglucosamine, is polycationic at acidic pH values and can form complexes with negatively charged molecules present either in solution or in the colloidal state, inducing their flocculation. Experiments in the pH range 2.0–7.0 showed that chitosan did not selectively precipitate peptides in the solubilized WISF, but fractionated WSE quite effectively, especially at pH 4.0. Analysis of the soluble and insoluble material obtained from WSE at pH 4.0 showed that the concentration of peptides, especially low molecular weight peptides, was much higher in the supernatant than in the precipitate, and there were large qualitative and quantitative differences between the peptide profiles of the two fractions. These results indicate that chitosan may offer an alternative to other methods (e.g. ultrafiltration or fractionation with ethanol) for the selective precipitation of water-soluble peptides in cheese prior to further analysis. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Chitosan is a derivative of chitin, a polymer of *N*-acetylglucosamine, which is a major component of the skeletal material of crustacean shells (Knorr, 1991). It is produced from chitin by deacetylation using alkali treatment at an elevated temperature. Chitosan and its derivatives are used in many industrial applications, including water- and food-processing; applications in foods have been reviewed by Shahidi (1994).

One of the main uses of chitosan in foods is based on its ability to flocculate lipids and proteins via the following mechanism: the pK of the amino group of glucosamine residues is about 6.3 (Muzzarelli, 1985) and hence chitosan is polycationic at acidic pH values and can form complexes with negatively charged molecules present in solution or in the colloidal state, inducing their flocculation. Hwang and Damodaran (1995) reported the use of chitosan to remove lipids from cheese whey. Whey protein concentrates (WPC) prepared by ultrafiltration usually contain 5–15% fat (Morr *et al.*, 1973); the addition of 0.01–0.02% chitosan to Cheddar cheese whey at pH 4.5 almost completely removed the milk fat globule membrane fragments prior to ultrafiltration (Hwang & Damodaran, 1995).

Many techniques for the separation of the nitrogenous components of cheese and the chemical characterization of cheese ripening exploit differences in the solubility of peptides in various reagents (Christiansen *et al.*, 1991; McSweeney & Fox, 1996). Crude fractionation (extraction and precipitation) techniques involve the use of water, salt solutions, acids or organic solvents. The objective of this work was to study the usefulness of chitosan for the selective precipitation of proteins and peptides in cheese.

MATERIALS AND METHODS

Materials

Samples from a 9-month-old Cheddar cheese were used.

Practical grade chitosan from crab shells (Sigma Chemical Co., St. Louis, MO, USA) was used as a 1% solution in 10% acetic acid.

Acrylamide, *N*,*N*′-methylene-bisacrylamide and *N*,*N*,*N*′,*N*′-tetramethylethylendiamine (all synthesis grade) were purchased from Merck (Darmstadt, Germany); trifluoroacetic acid (TFA) was from Sigma; tris(hydroxymethyl)aminomethane was from BDH Chemicals Ltd. (Poole, UK); acetonitrile (HPLC grade) was from Labscan Ltd. (Dublin, Ireland); Coomassie brilliant blue G25 was from Merck and bromophenol blue from Aldrich Chemical Company (Milwaukee, WIS, USA). Other chemicals used were of analytical grade.

*To whom correspondence should be addressed.

Methods

Preparation of the water-soluble and water-insoluble fractions of cheese

The water-soluble (WSE) and water-insoluble (WISF) fractions of Cheddar cheese were prepared by the method of Kuchroo and Fox (1982). Grated cheese was mixed with distilled water at a ratio of 1:2 (w/v) and homogenized in a Colworth Stomacher 400 (Seward Laboratory, UK) for 5 min at ≈20°C. After holding in a water-bath at 40°C for 1 h, the homogenate was centrifuged at 3000*g* for 30 min at 5°C to sediment the insoluble material. The supernatant was filtered through glass wool and Whatman No. 113 filter paper.

Fractionation of the water-soluble extract with chitosan

Chitosan was added to samples of WSE to a concentration of 0.02%, based on the observations of Hwang and Damodaran (1995), and the pH of subsamples was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0 or 7.0. The mixtures were held at room temperature for 10 min and then centrifuged at 3000*g* for 30 min. The resulting soluble and insoluble fractions were collected and processed separately.

In a second experiment, a sample of WSE containing 0.02% chitosan was adjusted to pH 7.0. The mixture was held at room temperature for 10 min and centrifuged at 3000*g* for 30 min. The precipitate was recovered and additional chitosan (to 0.01%) added to the supernatant to compensate for the loss of chitosan in the precipitate; the pH was adjusted to 6.0. The procedure was repeated at 1 pH unit increments to a final pH of 2.0.

Fractionation of the water-insoluble nitrogen with chitosan

A sample of WISF was dissolved at 10% in 0.1 M trisodium citrate buffer, pH 7.0. Two experiments were performed as for the WSE. In the first experiment, the pH was varied from 2.0 to 7.0; in the second experiment, the pH was reduced stepwise from 5.0 to 3.0 in 0.5 unit increments.

Urea–polyacrylamide gel electrophoresis (urea-PAGE)

Electrophoresis was performed in 4.5 M urea-containing polyacrylamide slabs (12.5% C, 4% T, pH 8.9) in a Protean II-xi cell connected to a Bio-Rad Model 1000/500 power supply (Bio-Rad Laboratories, Watford, UK) according to the method of Andrews (1983). Gels were stained directly by the procedure of Blakesley and Boezi (1977).

Gel filtration chromatography

Samples of WSE and the supernatant and precipitate obtained at pH 4.0 in the presence of 0.02% chitosan were chromatographed at 20°C on a 80 cm×2 cm column of Sephadex G-100 (Sigma) fitted to a Bio-Rad Econo System (Bio-Rad Laboratories, Hercules, CA, USA), using distilled water as eluent at a flow rate of 1 ml min^{-1}. Fractions (5 ml) were collected and the protein content was determined by measuring the absorbance at 280 nm.

High-performance liquid chromatography

The fractions from gel filtration were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a JVA C8 column (4.6 mm×25 cm, 5 μm particle size) (JVA Analytical Ltd., Dublin, Ireland) fitted to a Waters 626 pump, a Waters 600S controller and a Waters 717 autosampler (Waters Chromatography Division, Millipore Corp., Milford, MA, USA). Detection was at 214 nm using a Waters 486 detector connected to a NEC 466 computer. Chromatographic conditions were as follows: solvent A, 0.1% (v/v) TFA in deionized water (Milli-Q; Millipore); solvent B, 0.1% (v/v) TFA in acetonitrile. The samples were eluted first with 100% A for 5 min, then with a gradient from 0% to 50% B over 55 min, followed by 50% B for 6 min, then to 60% B over 4 min, followed by 3 min at 60% B. The flow rate was 0.75 ml min^{-1}. Samples were dissolved in 0.1% TFA and filtered through 0.45 μm cellulose acetate filters (Sartorius, Göttingen, Germany) for application to the column.

RESULTS

Urea-PAGE showed that chitosan gave good fractionation of WSE at pH 2.0, 3.0 and 4.0 (Fig. 1). The band patterns showed large differences between the supernatants and precipitates at those pH values. At pH 5.0, 6.0 and 7.0, most of the nitrogen of the WSE remained soluble in 0.02% chitosan and little fractionation was achieved. When the pH was reduced stepwise, the best fractionation was obtained at pH 4.0 (Fig. 2).

Fractionation of WISF using chitosan was not very effective. The addition of chitosan at different pH values did not influence the electrophoretograms of the supernantants, in all of which the same bands were present (results not shown). No precipitate was obtained at pH 5.0, 6.0 or 7.0; some precipitation was observed at pH 4.0 and copious precipitation occurred at pH 2.0 and 3.0, although the electrophoretograms of both supernatants and precipitates at pH 2.0 and 3.0 were very similar. When the pH was reduced stepwise, most of the WISF precipitated at pH 5.0 in 0.02% chitosan; some further precipitation occurred at pH 4.5 and 4.0, but very little additional precipitation occurred at pH 3.5 and 3.0.

Based on these results, further experiments were performed only on the fractionation of WSE with 0.02% chitosan at pH 4.0. WSE and the soluble and insoluble fractions thereof were chromatographed on Sephadex G-100. Since chitosan is insoluble at pH 7.0, it was removed from the supernatant and precipitate by adjusting the pH to 7.0. Figure 3 shows the elution

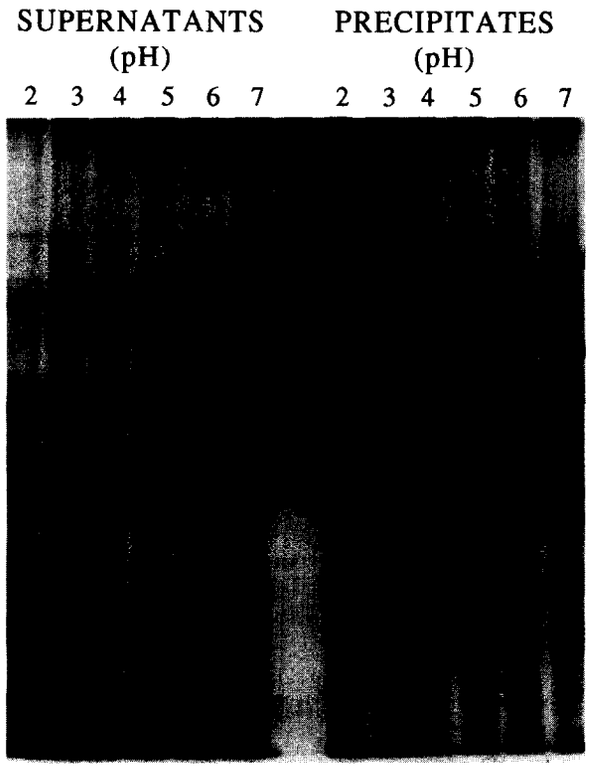

Fig. 1. Urea–PAGE electrophoretograms of the supernatants and precipitates obtained from the water-soluble extract of Cheddar cheese treated with 0.02% chitosan at pH 2.0, 3.0, 4.0, 5.0, 6.0 or 7.0.

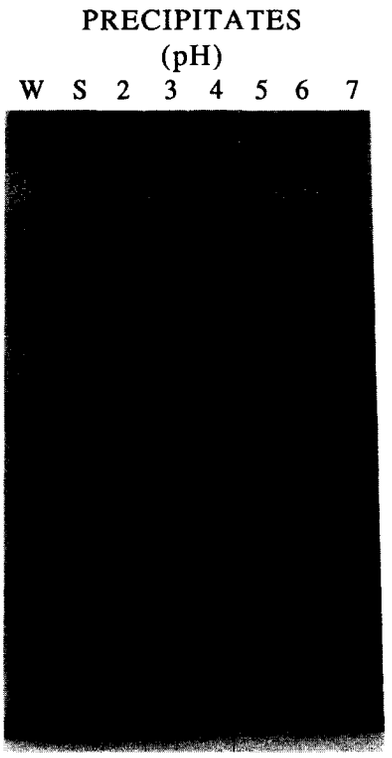

Fig. 2. Urea–PAGE electrophoretograms of the water-soluble extract of Cheddar cheese and the supernatant and precipitates obtained on reducing the pH stepwise from 7.0 to 2.0 after the addition of 0.02% chitosan. W, water-soluble extract; S, supernatant at pH 2.0.

profiles of the three samples. The 5 ml fractions were pooled into three groups, as indicated. The supernatant contained mainly peptides eluting between 180 and 250 ml (fraction III), while the precipitate was dominated by peptides that eluted in fractions I and II.

The fractions obtained by gel filtration were analysed by RP-HPLC. The chromatograms in Fig. 4 show that fraction I was the least complex of the three and the concentration of peptides was, in general, higher in the fractions of the supernatant. Qualitative and quantitative differences in the peptide profile of each fraction were observed (Fig. 4). Comparing the profile of fraction I of the supernatant and precipitate, two peaks (Fig. 4, fraction I, peaks 1 and 2) appeared only in the precipitate, and some peaks with retention times between ≈32 and ≈54 min (Fig. 4, fraction I, area A) appeared only in the supernatant. The two principal peptides in the supernatant, with retention times of ≈57 and ≈58 min, were also present in fraction I of the precipitate, but at lower concentrations (Fig. 4, fraction I, peaks 3 and 4). In relation to fraction II, some peaks eluting between ≈52 and ≈56 min were more significant in the precipitate (Fig. 4, fraction II, area B). Also, fraction II of the precipitate contained a high concentration of hydrophobic peptides (Fig. 4, fraction II, area C). There were major differences in the relative proportions of some peptides in fraction III of both supernatant and precipitate (Fig. 4, fraction III, peaks 5–9), while a slightly higher concentration of hydrophobic peptides was found in fraction III of the supernatant (Fig. 4, fraction III, area D).

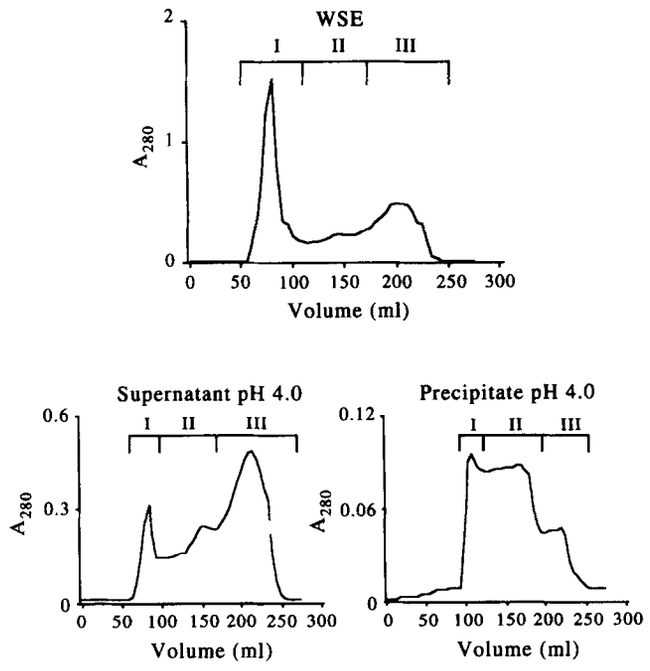

Fig. 3. Chromatograms of the water-soluble extract and the supernatant and the precipitate obtained from it after the addition of chitosan at pH 4.0 on Sephadex G-100. Eluent, distilled water; flow rate, 1 ml min^{-1}; temperature, 20°C.

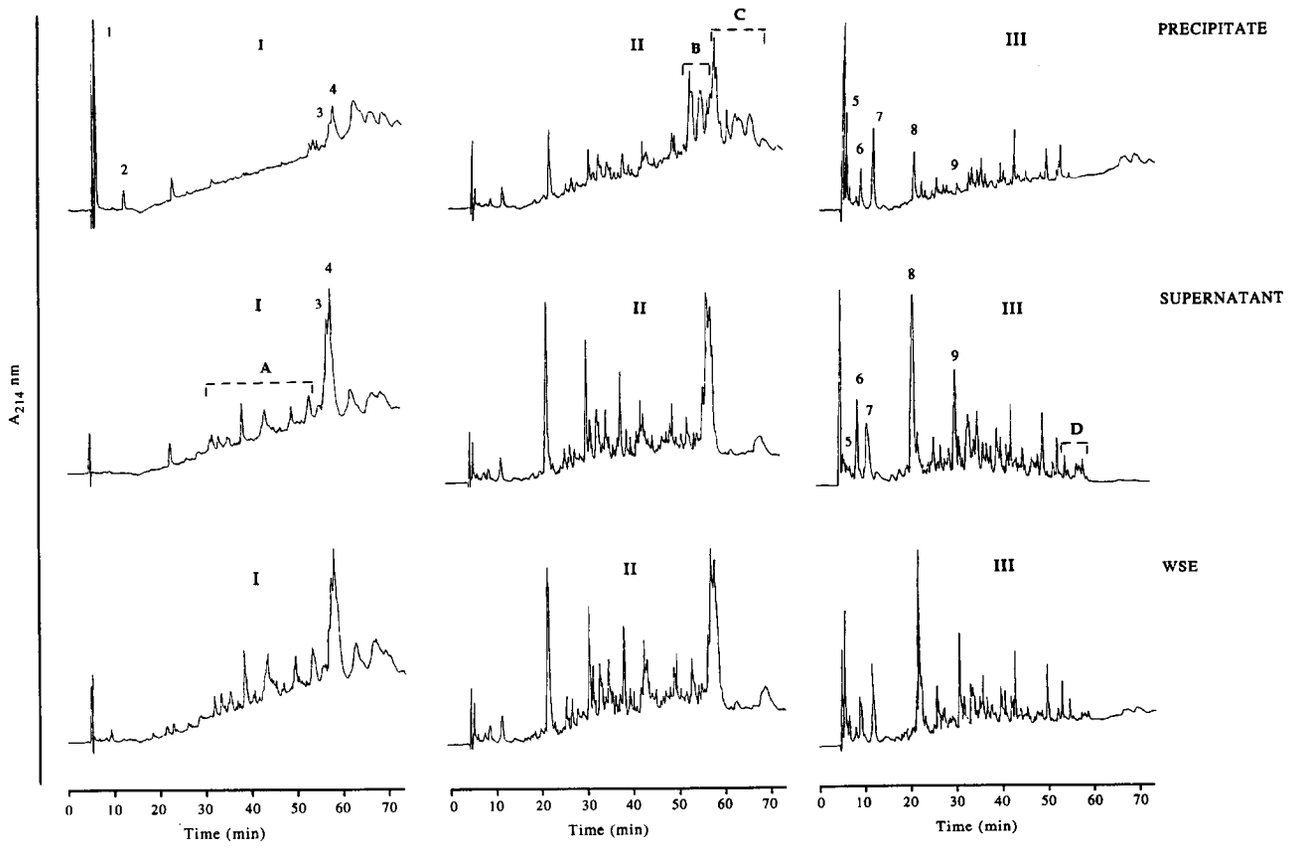

Fig. 4. RP-HPLC chromatograms of fractions I, II and III from Sephadex G-100 of the water-soluble extract and the supernatant and the precipitate obtained from it after the addition of chitosan at pH 4.0.

These results indicate that chitosan may offer an alternative to other methods (e.g. ultrafiltration, fractionation with ethanol) for the fractionation of water-soluble peptides in cheese prior to further analysis.

REFERENCES

Andrews, A. T. (1983). Proteinases in normal bovine milk and their action on caseins. *J. Dairy Res.*, **50**, 45–55.

Blakesley, R. W. & Boezi, J. A. (1977). A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G 250. *Anal. Biochem.*, **82**, 580–582.

Christiansen, T. M. I. E., Bech, A. M. and Werner, H. (1991). Methods for crude fractionation (extraction and precipitation) of nitrogen components in cheese. *Bulletin of the International Dairy Federation*. IDF, Brussels, pp. 4–9.

Hwang, D. C. & Damodaran, S. (1995). Selective precipitation and removal of lipids from cheese whey using chitosan. *J. Agric. Food Chem.*, **43**, 33–37.

Knorr, D. (1991). Recovery and utilization of chitin and chitosan in food processing waste management. *Food Technol.*, **45**(1), 114–122.

Kuchroo, C. N. & Fox, P. F. (1982). Fractionation of the water-soluble nitrogen from Cheddar cheese: chemical methods. *Milchwissenschaft*, **37**, 651–653.

McSweeney, P. L. H. and Fox, P. F. (1996). Chemical methods for the characterization of proteolysis in cheese during ripening. *Le Lait*, in press.

Morr, C. V., Swenson, P. E. & Richter, R. L. (1973). Functional characteristics of whey protein concentrates. *J. Food Sci.*, **38**, 324–330.

Muzzarelli, R. A. A. (1985). Chitin. In *The Polysaccharides*, Vol. 3, ed. G. O. Aspinall. Academic Press, New York, pp. 417–450.

Shahidi, F. (1994). Seafood processing by-products. In *Seafoods: Chemistry, Processing, Technology and Quality*, eds F. Shahidi & J. R. Botta. Blackie Academic & Professional, Glasgow. pp. 320–334.