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HYDROPHOBIC INTERACTION FAST PROTEIN LIQUID CHROMATOGRAPHY OF MILK PROTEINS

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SUMMARY

Bovine whey proteins and caseins were separated by hydrophobic interaction chromatography with the new Pharmacia fast protein liquid chromatography column, phenyl-Superose. Total casein was separated using a decreasing gradient of 0.8 to 0.05 *M* sodium phosphate and a constant 3.75 *M* urea concentration at pH 6.0. The order of elution of caseins was $\beta < \gamma, \alpha_{s2} < \kappa < \alpha_{s1}$, and β-casein was always eluted first. Whey proteins were separated with a decreasing salt gradient of 1.5 to 0 *M* ammonium sulphate in 0.05 *M* sodium phosphate at pH 7.0. The order of elution was β-lactoglobulin < bovine serum albumin < immunoglobulin < α-lactalbumin. The elution order of proteins from the column did not correlate with the calculated average hydrophobicities but the method was considered to be a measure of the "effective" hydrophobicity of proteins and therefore of more use for attempting to relate hydrophobicity to functional properties of proteins. The method shows significant advantages over conventional techniques allowing rapid optimization of elution conditions and reducing run times from 24 h or more to less than 2 h.

INTRODUCTION

Chromatographic methods based on differences in hydrophobicity between proteins and enzymes provide a useful alternative, or addition, to methods based on size (gel filtration) or charge differences (ion-exchange, chromatofocusing). There are two types of method for such experiments, reversed-phase chromatography and hydrophobic interaction chromatography (HIC). The former utilises high concentrations of organic solvents and often extreme (usually acid) pH values which generally lead to a complete loss of enzymic or other biological activity. The conditions employed in HIC are much less disruptive to protein structure and these methods therefore have many advantages when dealing with biological materials.

The use of fast protein liquid chromatography (FPLC) has revolutionised the separation of proteins in the laboratory. Both preparative scale and analytical work is now possible in a fraction of the time required using conventional chromatographic methods. Ion-exchange, reversed-phase, gel filtration and chromatofocusing columns

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have been available for the FPLC system for some time. However, until recently HIC was only possible using conventional column equipment or by high-performance liquid chromatography (HPLC) with the TSK Phenyl-5PW column. These have been used for the separation of proteins, including some of the whey proteins[1–3], although a separation from total whey has not been reported. Pharmacia Fine Chemicals have now introduced a new column of phenyl-Superose 12, the bonded hydrophobic phenyl groups enabling rapid HIC using their FPLC equipment.

Separation of milk proteins on the basis of molecular mass and charge has been achieved using gel filtration and ion-exchange columns on the FPLC equipment[4,5], but up until now only reversed-phase columns have been available for separation by hydrophobicity[6]. Reversed-phase HPLC has also been used for the separation of whey proteins[7,8] and caseins[9].

The purpose of this work was to assess the usefulness of the new hydrophobic interaction (HI-FPLC) column, optimizing separation conditions for both caseins and whey proteins. Bovine caseins have been separated using conventional HIC columns of phenyl and octyl sepharose[10] but these were not considered ideally suited to routine work due to their relatively low resolving power, low gel capacity and relatively long run times. Rapid HI-FPLC should alleviate these problems.

Current methods used for the determination of hydrophobicity, *e.g.*, calculation[11,12], partition methods[13] and fluorescent probe methods[14], are not wholly satisfactory when attempts are made to correlate hydrophobicity with functional properties[15]. HIC should be the best way of assessing the hydrophobic character of a protein in terms of its "effective" hydrophobicity.

EXPERIMENTAL

Materials

Total casein was prepared by isoelectric precipitation of bovine milk from a cow homozygous for β-casein A1 from the institute herd. Total whey protein was prepared from the acid whey fraction of bulk bovine milk, the individual whey proteins were purified by ion-exchange chromatography on DEAE-cellulose. Purified α_{s1}-, α_{s2}-, β- and κ-caseins were prepared using a similar method[16].

Instrumentation

The Pharmacia FPLC system (Pharmacia Fine Chemicals AB, Uppsala, Sweden) fitted with a phenyl-Superose HR 5/5 hydrophobic interaction column was used throughout.

Gradient hydrophobic interaction chromatography

For separation of both caseins and whey proteins various different buffer and salt gradient systems were evaluated.

Caseins. Buffer systems evaluated for caseins included decreasing salt gradients of ammonium sulphate, sodium sulphate, sodium acetate, potassium bromide or potassium thiocyanate in 0.05 *M* sodium phosphate or Tris–HCl buffers at various pH values in the range 6.0–9.0. Ethylene glycol (50%) was added to the final (low-salt) buffer in some cases. The most satisfactory system was found to be a 0.8 *M* sodium phosphate buffer gradient decreasing from 0.8 *M* to 0.05 *M* and containing

a constant 3.75 *M* urea concentration throughout, at pH 6.0. The casein samples (10 mg/ml) were dissolved in initial buffer (0.8 *M*) and solid urea added to give a concentration of 8 *M*, 500-μl samples were applied to the column.

Whey proteins. The buffer systems evaluated for whey proteins included ammonium sulphate, sodium sulphate, sodium acetate and sodium chloride salts in buffers similar to those used for casein separations. The final buffer system of choice was a 1.5 *M* ammonium sulphate salt gradient decreasing from 1.5 *M* to 0 *M* in 0.05 *M* sodium phosphate at pH 7.0. A volume of 500 μl total whey protein (10 mg/ml) dissolved in initial high-salt buffer was applied to the column.

Running conditions. The runs consisted of an initial 3-ml passage of high-salt buffer followed by the gradient (in 25 to 50 ml volume) and a 3-ml wash with salt-free buffer, then 3 ml of initial (high-salt) buffer to re-equilibrate the column. Flow-rates of 0.3–0.5 ml/min were used depending on the buffer composition and 0.5-ml fractions were collected throughout each run for identification of the peaks. Peak identities were established by running pure samples through the column under identical conditions and confirmed by slab polyacrylamide gel electrophoresis using T = 12.5%, C = 5% gels containing 4.5 *M* urea for casein analysis and no urea for whey protein analysis[16].

RESULTS AND DISCUSSION

It was hoped that both caseins and whey proteins would be separable using the same conditions, but this was not realised. The average hydrophobicities, calculated according to Bigelow[11] (Table I), of the two groups of proteins are similar but the proteins behave very differently on the HIC column. This was most probably because the whey proteins are globular proteins with a considerable amount of secondary and tertiary structure, whereas the caseins have a low degree of order in their structure[10].

Casein separation

The caseins are very hydrophobic and in a 1.7 to 0 *M* ammonium sulphate gradient in 0.05 *M* phosphate buffer at pH 7.0, they were bound so tightly to the column that it was difficult to remove them even when 50% ethylene glycol was added to the low-salt reservoir. This was also the case with a 0.8 to 0 *M* sodium acetate buffer gradient and a 1.0 to 0 *M* sodium sulphate buffer both at pH 8.0. In these cases all the caseins came off at the end of the gradient as one large peak or two partly resolved peaks. Urea (8 *M*) was used to remove the residual protein from the column. When potassium thiocyanate in Tris–HCl buffer, pH 9.0 was tried with 50% ethylene glycol in the low-salt buffer, a separation was achieved, but the first peak (β-casein) was not retained. A lower pH of 7.5, which increases the hydrophobic interactions between the sample and the column, shifted the elution profile to a higher concentration of final (low-salt) buffer by about 9% but the first peak was still not retained by the column. Omission of ethylene glycol from the final buffer did not change the elution profile significantly and was beneficial in reducing the column back pressure.

With potassium bromide as the salt generating the gradient, in a Tris–HCl buffer at pH 8.0 retention of the caseins on the column increased giving a good

separation of total casein. With this system the individual α_{2}-, κ-, γ- and α_{s1}-caseins adhered too strongly to the column and were poorly separated at the end of the gradient which therefore had to be held at 100% until elution of proteins was complete. The small peak eluting before the gradient began appeared to be some aggregated casein (the caseins associate in solution in the absence of urea).

To ensure that the caseins were completely dissociated and also to obtain a profile with all the peaks eluting within the region of the gradient, a urea-containing buffer was used. Urea is a chaotropic agent itself so clearly if made up in a buffer such as those containing potassium thiocyanate or potassium bromide the casein would not adhere to the column. Indeed this was found to be the case when the urea was made up in 1 *M* potassium bromide, 0.05 *M* Tris–HCl, pH 8.0. For this reason a less chaotropic salt, 0.8 *M* sodium phosphate in 3.75 *M* urea at pH 6.0 was chosen. The final bufffer was the same concentration of urea in 0.05 *M* sodium phosphate, pH 6.0 so that in this case the same constituent provided both the salt gradient and the buffering ions. An elution profile for the resulting separation is shown in Fig. 1 and as can be seen, all the casein components were well separated from each other in less than 2 h.

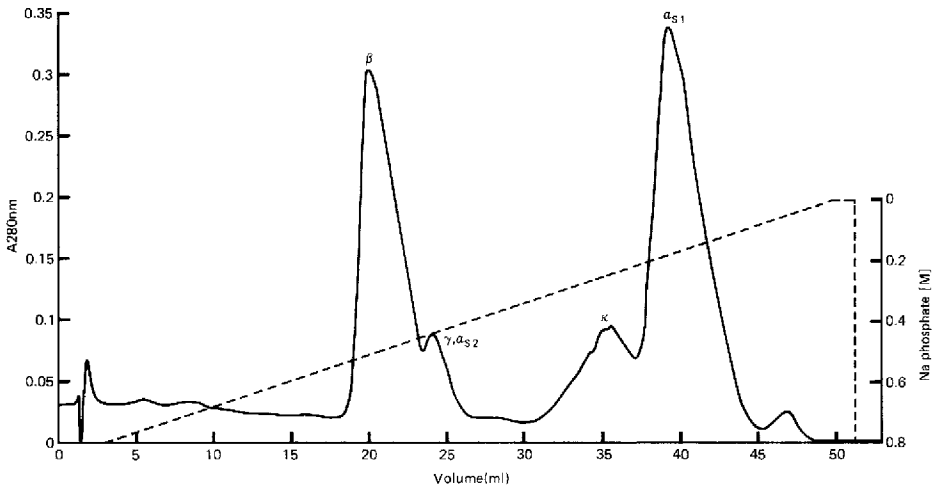

Fig. 1. HI-FPLC separation of total casein. A 500-μl sample (10 mg/ml) dissolved in 0.8 *M* sodium phosphate, 8 *M* urea, pH 6.0 was applied to the column and eluted at a flow-rate of 0.4 ml/min using a 0.8 to 0.05 *M* sodium phosphate gradient (broken line) in 3.75 *M* urea (pH 6.0), 3 ml after sample application. Peaks: β = β-casein; κ = κ-caseins; α_{s2} = α_{s2}-caseins; α_{s1} = α_{s1}-casein; γ = γ-caseins.

Whey protein separation

Although easier to separate than the caseins on this column the resolution was not found to be as good. Separations were achieved with gradients of phosphate and sulphate salts but with sodium acetate at pH 8.0 the whey proteins passed straight through the column. The elution profile obtained with a 1.5 to 0 *M* ammonium sulphate gradient in 0.05 *M* sodium phosphate (pH 7.0) buffer is shown in Fig. 2. The β-Lactoglobulin (β-lg) was resolved as a sharp peak but with all conditions tried,

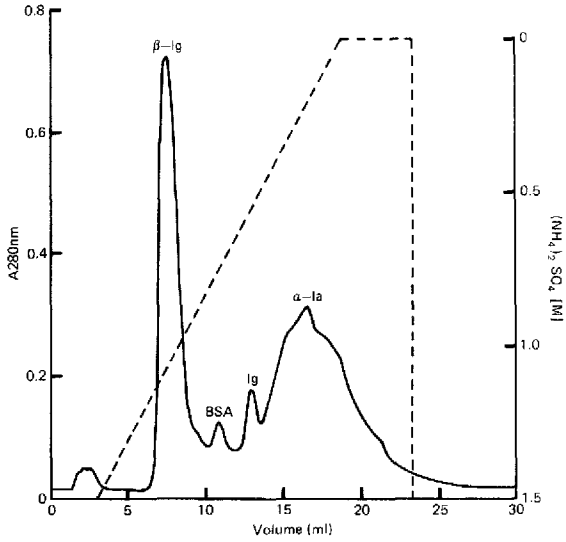

Fig. 2. HI-FPLC separation of total whey. A 500-μl sample (10 mg/ml) dissolved in 1.5 *M* ammonium sulphate, 0.05 *M* sodium phosphate buffer at pH 7.0 was applied to the column and eluted at a flow-rate of 0.5 ml/min using a 1.5 to 0 *M* sulphate gradient (broken line), 3 ml after sample application. Peaks: β-lg = β-lactoglobulin; BSA = bovine serum albumin; Ig = immunoglobulins; α-la = α-lactalbumin.

α-lactalbumin (α-la) eluted as a much more broad and less smooth peak. This is probably due to the molecular shape. With a more shallow gradient than that shown in Fig. 2, β-lg and bovine serum albumin (BSA) were completely resolved.

Although a separation of whey proteins was achieved with sodium sulphate, this salt was considered unsuitable for fractionation of whey by HIC. Uneven traces were obtained which were thought to be due to the protein salting-out on the column.

Relative hydrophobicities of the proteins

The relative retention of the caseins on the HI-FPLC column is $\beta < \alpha_{s2}$, $\gamma < \kappa < \alpha_{s1}$ (Table I). This order does not agree with the calculated order of average hydrophobicities. Kesharavarz and Nakai[15] also found no significant relationship between the calculated average hydrophobicity (Bigelow number) and elution order of whey and other proteins. In our work, β-casein eluted before the α_{s1} casein in all salt and buffer systems studied. Similar findings have also been reported by Creamer and Matheson[10]. They considered that one or more regions in the α_{s1} casein molecule adopt a conformation that can interact strongly with the hydrophobic groups on the HIC column and suggest that one such region may be residues 14–24 of the α_{s1}-casein B polypeptide chain. Also, the lower charge and higher average hydrophobicity of β-casein compared to α_{s1}-casein causes a strong tendency towards clustering of hydrophobic and hydrophilic residues[17] so the apolar residues of β-casein are likely to be more buried and consequently less accessible for interactions with the column matrix than those of α_{s1}-casein. Barrefors *et al.*[6] found that with reverse phase chromatography of caseins all the caseins except κ-casein eluted in order of their average hydrophobicity ($\kappa < \alpha_{s2} < \alpha_{s1} < \beta$). They suggested that the amphiphilic glyco-

TABLE I

COMPARISON OF AVERAGE HYDROPHOBICITIES AND RELATIVE MOBILITIES ON THE HYDROPHOBIC INTERACTION COLUMN, FOR CASEINS AND WHEY PROTEINS

Sample	*Average hydrophobicity* (cal/res)*	*Retention time on HI column***
α_{s1}-Casein	1170	0.79
α_{s2}-Casein	1096	0.48
β-Casein A_1	1316	0.40
κ-Casein A	1199	0.71
β-Lactoglobulin B	1199	0.28
α-Lactalbumin	1087	0.64
Bovine serum albumin	1085	0.42
Immunoglobulin	–	0.50

* Calculated according to Bigelow[11] (cal/res = calories per residue).

** Data for caseins taken from Fig. 1. Data for whey proteins taken from Fig. 2 (retention time = elution time/total run time).

macropeptide part of the κ-casein molecule was exposed and that HIC is strongly dependent on the buffer system and column material used.

The order of elution of whey proteins also was not the same as their calculated average hydrophobicities. The order of elution of the whey proteins was β-lg < BSA < Ig < α-la while the order of average hydrophobicities was (BSA < α-la < β-lg). Goheen and Englehorn[1] found that β-lg and BSA eluted as broad peaks, at almost identical retention times but in our system β-lg was eluted just before BSA and they were clearly separable. Using a TSK Phenyl-5PW column they reported that addition of methanol to the buffers improved the sharpness of the BSA peak[1]. In agreement with this we also found that inclusion of 10% methanol in buffers gave a small but not very significant improvement in the sharpness of BSA and α-la peaks, but in casein separations the peak profile was unaffected.

In conclusion, HI-FPLC has been shown to be far more rapid than conventional hydrophobic interaction chromatography for separation of proteins, reducing run times from about 24 h to 1 h or less and facilitating optimization of separation conditions. In general, the sharpness of the peaks obtained with this column was not as good as the profiles obtained with reversed-phase chromatography columns or the TSK Phenyl-5PW type of HIC column although far better than with conventional octyl- and phenyl-Sepharose columns.

This method showed potential for measurement of the effective hydrophobicity of proteins, even though the relative retention times of caseins and whey proteins did not correlate with average hydrophobicity calculated from Bigelow number, a conclusion also reached with different proteins by other workers[6,10,15]. However relative retention times obtained by HIC are probably a better indication of the "effective hydrophobicity" of the protein and likely to be more useful when attempting to relate hydrophobicity to functional properties of proteins. The difference in elution behaviour between caseins and whey proteins showed that effective hydrophobicity de-

pends on the flexibility and conformation of the protein molecules as well as composition.

The elution behaviour of proteins could be altered by small changes in buffer composition. Increased pH, decreased temperature, increase in chaotropic nature of salt and changing to a non-polar solvent all decreased hydrophobic interactions between the protein and the column matrix. The finding that so many factors influenced the strength of hydrophobic binding confers great potential versatility on HIC as a separation method but optimization of separation conditions when so many different parameters are involved would often be prohibitively time consuming by conventional HIC methods. Therefore the combination of suitable columns with apparatus for high speed analysis, such as FPLC and HPLC, has very considerable advantages over earlier procedures.

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