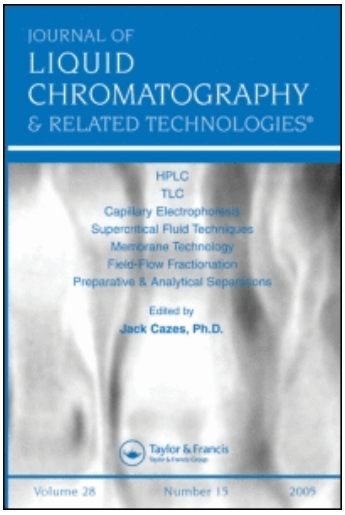

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High Performance Size Exclusion Liquid Chromatography of Small Molecular Weight Peptides from Protein Hydrolysates Using Methanol as a Mobile Phase Additive

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HIGH PERFORMANCE SIZE EXCLUSION LIQUID CHROMATOGRAPHY OF SMALL MOLECULAR WEIGHT PEPTIDES FROM PROTEIN HYDROLYSATES USING METHANOL AS A MOBILE PHASE ADDITIVE

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ABSTRACT

The separation of low molecular weight peptides according to their molecular weight has been a challenge. The earlier works using chaotropic additives in the mobile phase such as SDS or guanidium chloride failed to give a linear response for the semilogarithmic graph of molecular weight versus retention time. Here we report a mobile phase composition suited to the size exclusion separation of the peptides of molecular weight between 6000 and 250 on a TSK-SW 2000

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column. The mobile phase consists of a phosphate buffer 50 mM containing 0.1% TFA and 35% methanol. The different elution parameters and systematically investigated and with the optimum conditions, correlation value of 0.97 could be obtained when 6 standard protein and 16 peptides in the range of 66 000 to 250 daltons were tested. The usefulness of this technique is evaluated on the group separation of different protein hydrolysates according to their molecular weight. Moreover, the free amino acids could be completely separated from the peptides in the hydrolysates.

INTRODUCTION

The first criterion in the characterisation of protein hydrolysates destined for dietetic utilisation is their molecular weight (MW) distribution. The HPLC in the size exclusion mode is reported for proteins (1,2,3). However, the use of HPLC in the field of peptides separation is generally restricted to reverse phase. Although reasonable resolution is often obtained by using reverse phase HPLC, a complete characterisation of unknown protein hydrolysates is rather elusive.

It has been shown that protein hydrolysates should be rich in low molecular weight peptides with as little as possible free amino acids, for the hydrolysate to be of a high nutritional and therapeutic value (4).

Until now the soft gels like the Sephadex^{R} or Biogel^{R} have been used for the gel filtration of peptides of the protein hydrolysates(5). However, apart from their poor

mechanical properties, their inclusion limits do not allow the separation of small peptides.

Recently the silica based gels chemically bonded with hydrophilic compounds are commercially available under the name TSK-Gel Sw (Toyosoda, Japan) for the resolution of proteins according to their molecular weights (1,2,3). The ranges of separation reported for these gels are rather high and no separation could be achieved for peptides of MW 5000 (6). This is due to the fact that peptides of the same mass, but with different composition may exhibit a wide range of electrostatic and hydrophobic interaction with the low porosity columns, which leads to non-linearity in the retention behaviour. Even the elutions under denaturing condition, with the addition of SDS or guanidium chloride in the mobile could not allieviate this problem (7,8).

So, it was necessary to investigate the optimal gel pore size and the suitable mobile phase composition to have an exclusively size exclusion mode of separation to estimate the MW distribution of protein hydrolysates.

We here report the methodology for the successful determination of the MW distribution of protein hydrolysate, composed principally of peptides of MW $<$3000 by using the TSK-SW commercial column.

EXPERIMENTAL

The chromatographic equipment used consisted of a LKB pump model 2150 with a 20μl injection loop and a variable

wavelenght detector, routinely set at 214 nm and a HP integrator recorder model 3390 A Integrator, (Hewlett Packard). The column used is a 7.5 mm x 600 mm TSK-SW 2000 fitted with a precolumn Ultropac TSK-GSWP 7.5 x 75 mm. All the mobile phases tested contained mainly of 0.05 M phosphate solution adjusted to pH from 5.0 to 8.0. All the other reagents such as TFA, methanol, etc. were of HPLC grade. Prior to use all solvents were filtered with 0.45 millipore filter and degazed with ultrason. The flow rates were 0.7 to 1.0 mL/min and the pressure 40 to 60 bars. The system was maintained at constant temperature by keeping the solvents in a water bath.

The proteins and peptides used as standards are:

Proteins	M.W.
Bovin section albumin (BSA)	66 000
Ovalbumin	45 000
Peroxidase	40 000
B.Lactoglobulin	35 000
Ribonuclease	13 700
Cytochrome C	12 000

Peptides	M.W.
1. Trypsin inhibitor	6 700
2. ACTH	4 390
3. Insulin B chain	3 480
4. Polymixin sulfate	1 447
5. Bacitracin	1 411
6. Hys-Lys	395
7. Ala-Gly-Ser.L.Glu	362

8. Hexa Gly	360
9. Leu-Leu-Leu	357
10. Lys-Lys	347
11. Oxydised glutathion	307
12. PentaGly	303
13. Leu-Leu	244
14. Gly-Gly-Leu	245
15. Ala-Pro-Gly	243
16. Gly-L-Lys	240

The casein hydrolysates are kind gifts from Sopharga (France)

RESULTS

On most commercially available HPSEC columns run with aqueous mobile phases such as phosphates etc, the correlation between the retention volumes and the MW of small peptides is very poor. This is due to the differences in the nature and magnitude of different interactions such as ionic, hydrophobic and coulombic forces of the peptides of different composition, but of the same mass, with the high density (low porous) gel filtration medium such as TSK-SW 2000, columns.

Mobile phase additives such as phosphoric acid, Triethyl amine (TEA), Trifluoroacetic acid (TFA), are already reported to be efficient in the peptide separation by reverse phase mode (9,10). On a first attempt we made a systematic study of different aquous mobile phase compositions and calculated the correlation for the sixteen

Table 1. The correlation between retention volume vs log MW with different mobile phase conditions.

No	Gradient system	Correlation for 16 peptides
1	Phosphate buffer pH 8.0 → pH 5.0	0.65
2	Phosphate buffer pH 8.0 → pH 5.0 + 0.1% TFA	0.71
3	Phosphate buffer pH 8.0 → pH 5.0 + 0.1% TEA	no correlation and recovery is not total
4	Phosphate buffer pH 8.0 → pH 5.0 + 0.1% phosphoric acid	-id-
5	pH 8.0 → pH 5.0 + 0.1% HCl	-id-

standard peptides as described in the experimental section. The results are shown in Table 1.

It is observed that only 0.1% TFA seems to be suitable as an ion pairing additive to a phosphate buffer mobile phase to improve the correlation between the retention volume and the MW of these low MW peptides.

To further improve the correlation, we have tried an isocratic elution with a 0.05 M phosphate solution at pH 5.0 containing 0.1% TFA, hence a final pH of 2.5, with different

Table 2. The correlation between retention volume and log MW with varying concentrations of methanol in the mobile phase.

CH_3OH conc	R value
0%	0.60
10%*	0.85
20%*	0.89
25%*	0.90
30%	0.95
35%	0.97

* The homopolymers could not be easily identified.

concentrations of added methanol, as the mobile phase. The solvent is kept at +4°C in order to avoid any evaporation problem of this semi volatile aqueous organic buffer. The results are compiled in Table 2.

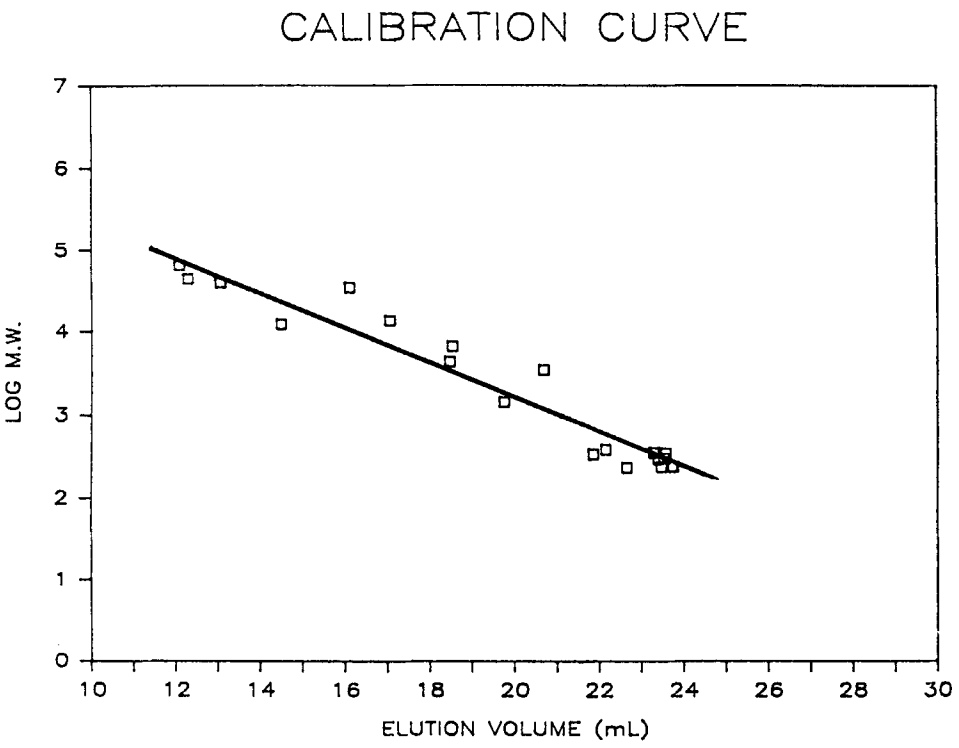


Figure 1.

The semilogarithmic plot of molecular weight vs retention volume for the standard proteins ans peptides listed in the experimental section.
Mobile phase: 0.05 phosphate buffer at pH 5.0 + 35% methanol + 1.0% TFA.

From these results, addition of 35% MeOH in the mobile phase containing phosphate buffer + 0.1% TFA seems to be the best suited for the separation of peptides according to their molecular weight. We have to note that the difference in R value (correlation for 30% and 35% methanol) is almost negligiable. From the semilogarithmic standard curve it could be seen that a linear correlation could be

obtained for a wide range of molecular weight from 66 000 to 200 with a correlation value of 0.97. (Fig 1).

With this mobile phase composition (35% MeOH) the different parameters such as flow rate, sample capacity, solute-solute interactions and sample preparation are determined.

- Flow rate: only two flow rates, 1 mL/min and 0.75 mL/min were tested because of the maximum pressure limitations set by the column suppliers. No change in the peptides retention could be seen. This is in accordance with the results of Rokushika et al. (12), that the HETP did not vary for small molecules on a TSK-Gel 2000 SW column as a fonction of flow rate.

- Sample preparation: The presence of salt (NaCL) in the injected sample (20 µl) affects the retention. It gives a parasite peak at 33 min, which is at the same position as di and tri peptides. Hence, the samples are best prepared in water or the elution buffer.

We have calculated the specific resolution factor (Rs) for certain pairs of small molecular weight peptides according to Kato et al. (7) using the equation:

$RS = 2(V_2 - V_1)/(W_2 + W_1)(\log M_1 - \log M_2)$.

Where V, W and M are the elution volumes, the peak width at the bottom and the molecular weight respectively. From these values it is found that the highest separation efficiencies are found in the molecular weight range of 2000 and 200.

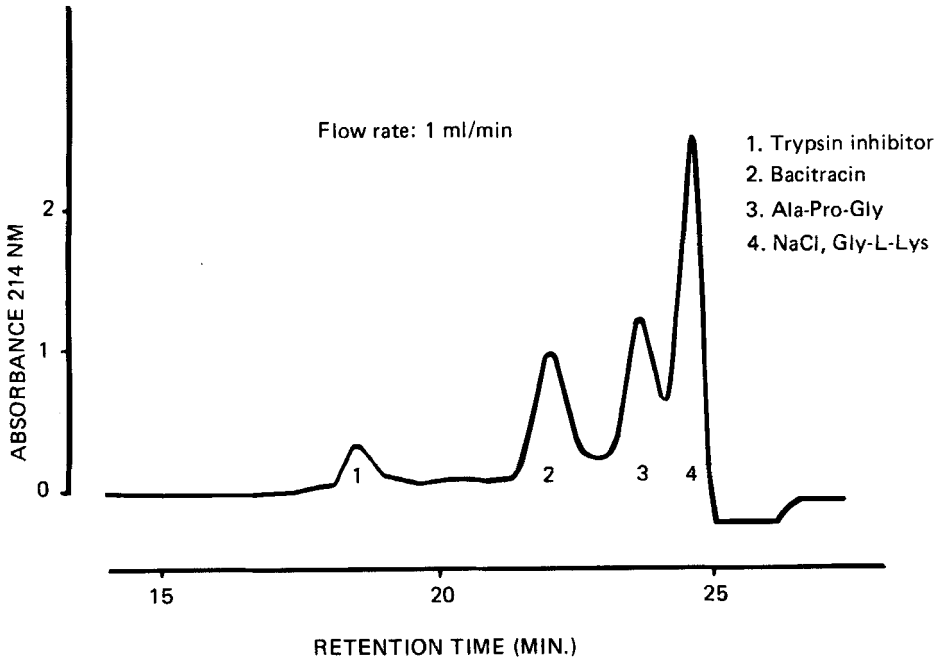


Figure 2.

The elution pattern of the mixture of pure standard peptides: The mobile phase composition is same as in Fig. 1.

As this investigation is oriented towards the applicability of this technique for the group separation of peptides from conplex, protein hydrolysates, according to their molecular weight distribution it was necessary to study the influence of peptide mixtures on the retention volumes, as compared to a single peptide in the sample, as in the case for the determination of the calibration curve. For this, we have made an artificial mixture of the standard peptides in the sample and the Fig.2 shows that peptides whether injected

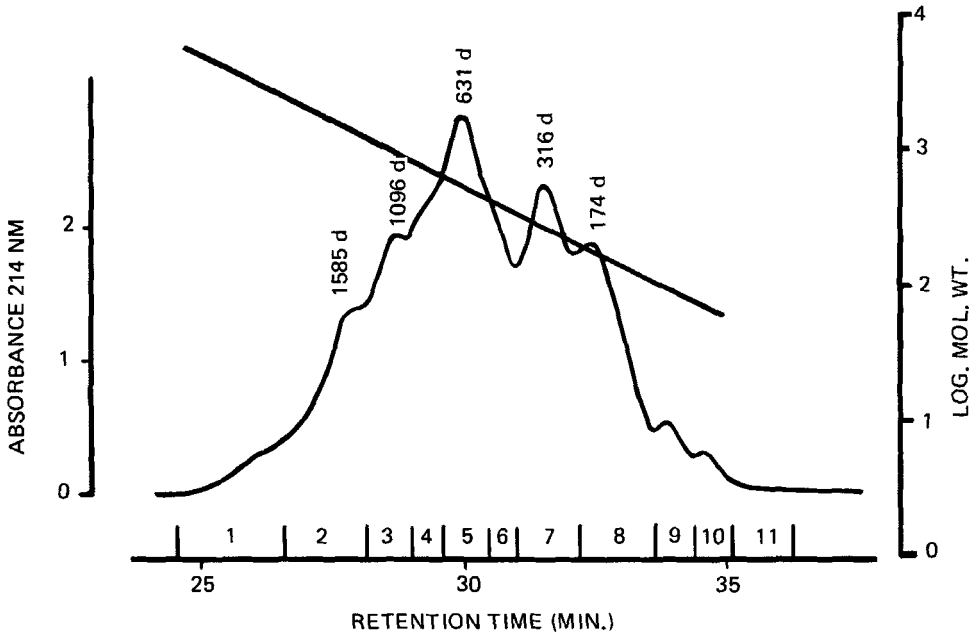


Figure 3.

Elution profile of a casein hydrolysate. Mobile phase same as in Fig. 1.

alone or as a mixture have the same retention volume and the elution profile.

The usefulness of this technique is demonstrated in the determination of the MW distribution profile of peptides, from a casein hydrolysates, as shown in Fig. 3, The sample consists mainly of peptides of MW from 2000 to 300, as expected by the hydrolysis conditions used by the manufactures. It was necessary to study the influence of sample load on the peptide resolutions, in order to use these fractions directly on a second dimension HPLC. It is

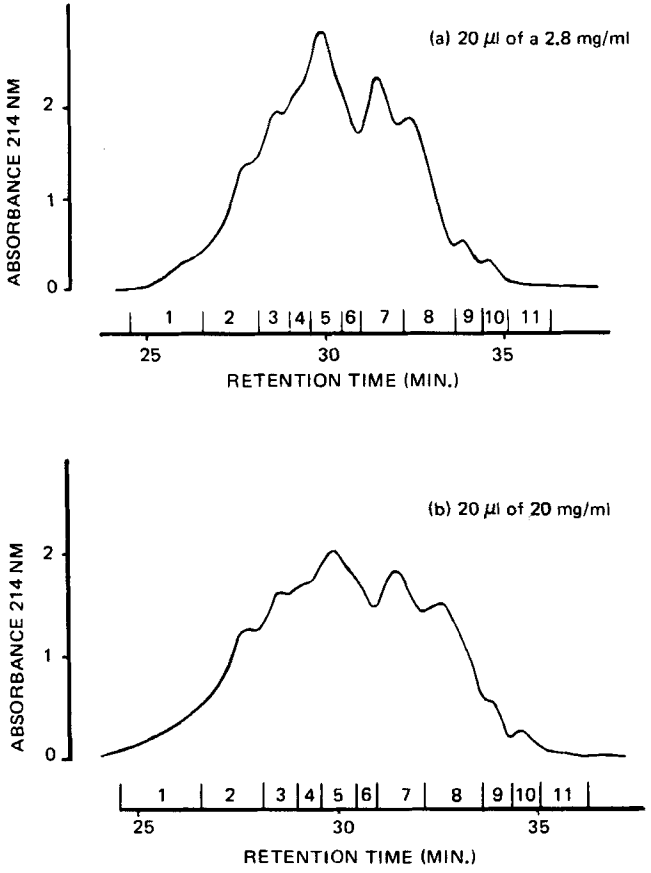


Figure 4.

Effect of the sample load in the form of a casein hydrolysate on the elution profile.

(a) 20 µl of a 2.8 mg/mL

(b) 20 µl of 20 mg/mL

(c) 20 µl of 50 mg/mL

All samples are dissolved in water and the mobile phase is the same as in Fig. 1.

found that up to 400 ug of the casein hydrolysate the resolution is unchanged; above this concentration no seperation was observed (Fig. 4a,b,c).

- A good reproductibility of the elution profile for the hydrolysate is obtained as verified both by the reinjections of the eluted peaks and by loading the same sample during 31 runs. The mean deviation for the retention time for the two peaks 5 and 7, are + 0.16 and + 0.13. The mean deviation for the peak area for peak 7 is 2.89.

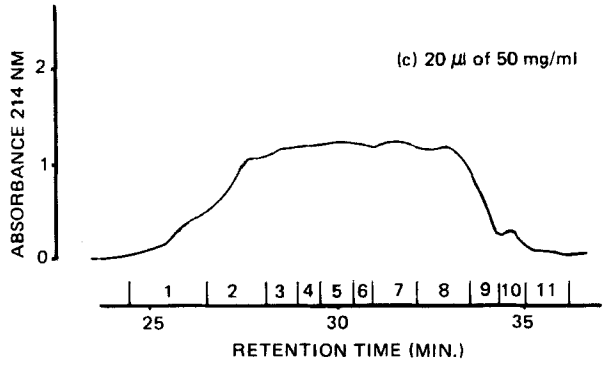
The free amino acids could be clearly separated from the peptides; thus, the amino acids such as cysteine, methionine, aspartic acid, glycine and tryptophane were run separately and with the peptide peak 7 from the hydrolysate and their retention time is always highter than 36 min whereas all the peptides are eluted before 32 min (Fig. 5).

This technique seems extremely promising and has the advantage of peptide recovery without encountering the problems of SDS removal as in the case of previous works.

Moreover, this technique is proved useful for monitoring the controlled hyodrolysis of the vegetable or animal proteins using free or immobilised proteases as shown in the example in Fig.6

DISCUSSION

Kato et al.(7) reported a separation range for TSK-SW 2000 column between 67 000 (BSA) and 5 000; using NaCl as the



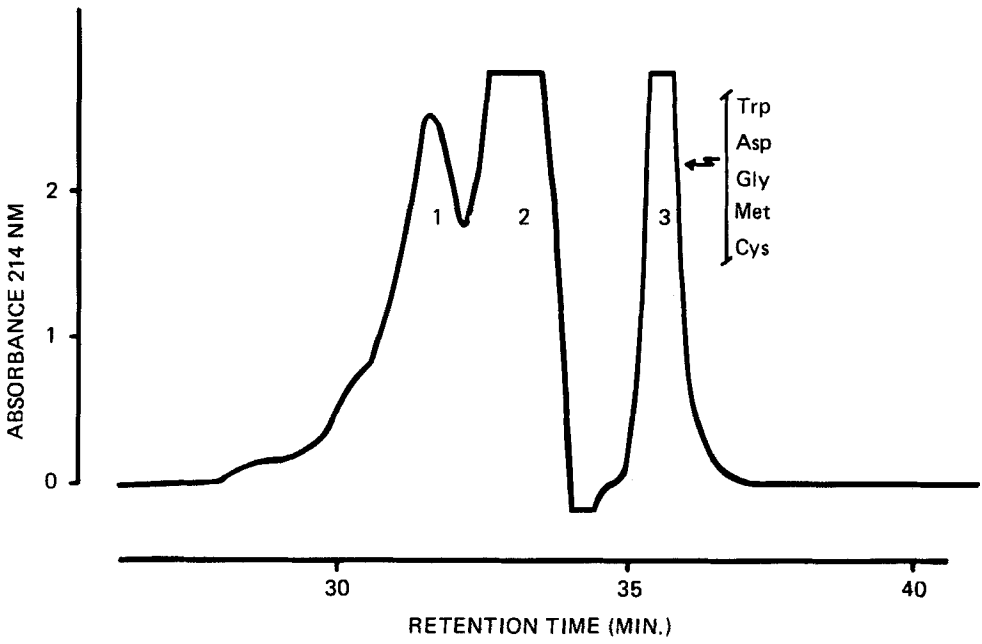


Figure 5.

Peptide peaks from a casein hydrolysate mixed with free amino acid.

Elution conditions as in Fig. 1.

Peak 1: peptide of MW 630, peak 2: $\simeq$ 300 and peak 3: free amino acid.

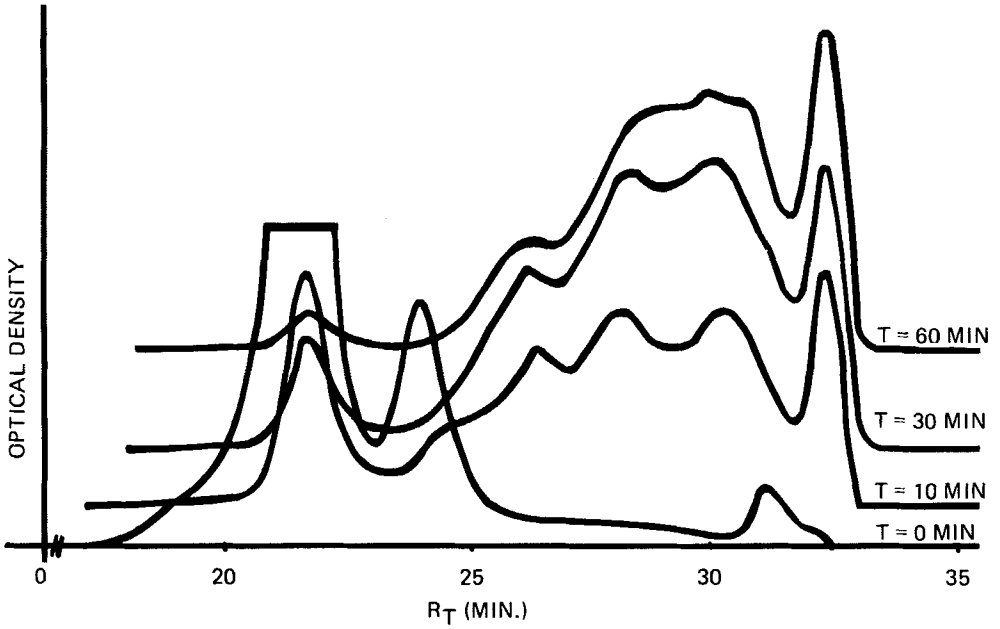


Figure 6.

Elution profile variations according to the degree of proteolysis of a milk protein.

mobile phase additive to a phosphate buffer. We could extend the range from 67 000 to 200 with a linear regression correlation between the retention volums and log. MW equal to 0.97 with a slope of - 5.9, by using the present mobile phase composition. Moreover it is to be noted that the total column volume with a 75 mm x 600 mm column is 26.5 mL. The fact that the retention volumes observed in the 67 000 to 200 MW range, using our mobile phase composition are between 12.1 mL and 27.0 mL indicates that we are operating on an exclusively gel filtration mode, where all the possible non specific adsorption of the peptides to the TSK column is overcome by this solvent composition where as when

0.3 M NaCL is used to over come the interactions the elution volume range reported are from 25.0 mL to 35.0 mL which clearly indicated the presence of adsorption phenomena (3). Moreover, more than 50% of the total column volume is used in our HPSEC separation of proteins and peptides, which implies that we can use smaller length columns for the same separation efficiency. The specific resolution factors (Rs) are calculated for certain pairs of small molecular peptides and a maximum separation efficiency could be obtained for peptides between 2000 and 200. Whereas the previous works (12) mentioned the maximum separation efficiency below 10 000.

Others additives such as Na_2SO_4 were also used to alleviate the non-specific retention of lysozyme on this column (1). However all these additives were not efficient in totally over coming the non-specific adsorption of small molecules such as peptides, aromatic amino acids and nucleotides (1) which we believe are due to the hydrophobic and hydrogen bond forces as interaction mechanisms as has been reported earlier for the soft gels (12).

Hefti (11) used ethyl alcohol as a mobile phase additive for the size exclusions chromatography of proteins. However, this system is not efficient for the separation of small peptides. Methyl alcohol seems to be better in overcoming the non-specific adsorption. This may be due to the better proton donor properties of the methyl alcohol, as the adsorption of the peptides to these columns may be explained in terms acceptor-donor interaction of peptides with the gel surfaces, which give rise to hydrogen bound forces.

CONCLUSION

This HPSEC technique is a very valuable tool not only for the determinations of the molecular weight distribution profile of unknown protein hydrolysates, but also for the follow up of proleolysis of proteins of both vegetable and animal origin.

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