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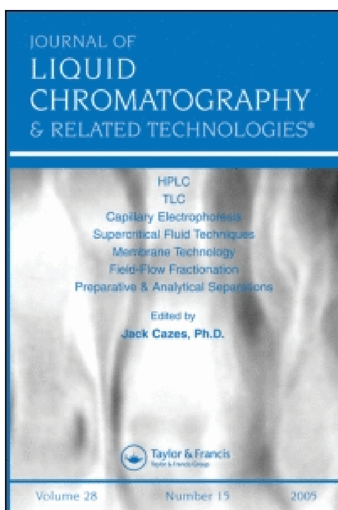

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ANALYSIS OF RAT CASEINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The analysis of rat caseins by high performance liquid chromatography is described. These procedures have confirmed the structural similarity of the polypeptide chains of native and dephosphorylated rat β_1- and β_2-caseins as well as to demonstrate that these two related proteins are structurally unrelated to the rat α_1-casein protein. In addition, these HPLC techniques allow the analysis of the phosphorylation patterns of these rat caseins following digestion with potato acid phosphatase (E.C. 3.1.3.2).

INTRODUCTION

The caseins, the major protein components of milk, are a family of relatively low-molecular weight phosphoproteins which in milk are found associated together in the form of protein

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micelles [1]. Whole rat casein contain three major casein components [2,3], the most abundant component (designated in this study as α_1-casein)has an apparent molecular weight of 43,000 daltons and represents over 50% of the total casein proteins. Another major fraction, with an apparent molecular weight of 28,000 daltons as determined by SDS-gel electrophoresis, can be resolved on DEAE-cellulose into two components in approximately equal amounts (designated β_1 and β_2). In a detailed study on these rat caseins, Hobbs and Smith [2] have proposed that the 28,000 dalton rat caseins are probably derived from the same polypeptide, differing only in the number of bound phosphate groups per molecule, and that both these rat β-caseins are unrelated structurally to the 43,000 dalton rat α_1-casein. Recently, we reported methods [4-6] for probing protein homologies based on enzymatic mapping analysis, using reversed phase high performance liquid chromatography (HPLC). In this paper we wish to describe the application of these methods to the analysis of rat α_1-, β_1- and β_2-caseins which not only confirms the proposed differences between the α_1 and β proteins but also allows a direct comparison of the phosphorylated and dephosphorylated rat caseins.

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MATERIALS AND METHODS

Rat Whole Casein Isolation and Fractionation.

Milk was collected from female rats of the Wistar strain between 9 and 14 days post partum following anaesthetisation with ketamine hydrochloride (50mg/ml, 0.5ml i.p.) and administration of oxytocin (5 I.U. i.p.). Whole casein was prepared by centrifugation at 20,000g for 1h of the diluted milk (1:2) in the presence of 75mM $CaCl_2$, the final pellet was dissolved in 100mM EDTA, pH 7.6, exhaustively dialysed against de-ionised water and stored at -20°. The separation of the rat casein

components on DEAE-cellulose essentially follow procedures described [7] for bovine casein. In brief, the crude rat whole casein (100mg) was chromatographed on a DEAE cellulose column (30 x 1cm) using an initial buffer containing 6M urea, 10mM Tris-HCl, pH 8.6, 1mM EDTA and 1mM DTE. The casein components were eluted with a linear gradient of 0 to 220mM NaCl in the same buffer. Appropriate fractions were pooled, dialysed against 10mM NH_4HCO_3, lyophilised and analysed by SDS-gel electrophoresis using a modification of the procedure of Laemmli [8].

Casein Dephosphorylations.

The rat caseins were dephosphorylated by a procedure similar to that of Bingham et al. [9] using potato acid phosphatase (E.C. 3.1.3.2). The caseins were dissolved in a buffer containing 50mM imidazole-HCl, pH 7.0, at a concentration of 1-3mg/ml and the potato acid phosphatase (60units/mg) added at an enzyme/casein ration of 1:1000. The solution was applied to a Sephadex G25 column in the same buffer and incubated at 37° for 2.5-3h. The proteins were then eluted and desalted on a second Sephadex G25 column equilibrated in 10mM NH_4HCO_3 and lyophilised.

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Enzymatic Digestion of Caseins.

The trypsin digestions were carried out in 100mM NH_4HCO_3 pH 8.5, 1mM DTE using an enzyme (1mg/ml in 1mM HCl) to protein ratio of 1:100. After incubation for 2h at 37°, a further aliquot of trypsin was added, incubation continued for a further 2h and the samples analysed immediately by HPLC or frozen at -20°.

High Performance Liquid Chromatography.

The configuration of the HPLC equipment, sample and solvent preparation, which were used in this study, are essentially as described previously [4-6]. Waters μBondapak Fatty Acid

Analysis columns (30 x 0.39cm) were used throughout, at a flow rate 2.0ml/min, with a linear 60min gradient generated from water-16mM H_3PO_4 to 50% acetonitrile-50% water-16mM H_3PO_4. Peptides were detected by their absorbance at 210nm.

RESULTS AND DISCUSSION

Compared to conventional techniques of peptide separation and analysis, it is now possible to obtain superior peak resolution for both hydrophilic and hydrophobic peptides with modern reversed-phase HPLC approaches. In addition, these methods offer short elution times and, frequently, excellent recoveries. In previous papers [4-6,10,11] from this laboratory, we have exploited these advantages in a variety of studies on the analysis and isolation of peptides from synthetic and natural sources. An additional area, where the potential of these methods has become increasingly evident, is their use in the separation of peptide fragments derived from proteins following enzymatic digestion thus allowing the comparative profiling of different proteins, either by way of assessment of the homogeneity or structure, or by the detection of distinctive sequence homologies of related proteins. All of these advantageous features were apparent in the HPLC analysis of the different rat caseins.

Typical elution profiles for the 2h tryptic digests of the phosphorylated and dephosphorylated rat α_1-, β_1- and β_2-caseins are shown in Figs. 1-3 respectively. No significant changes in these elution profiles were observed when the trypsin digestion was carried out for 4h. Comparison of the elution profiles of the tryptic peptides of the dephosphorylated forms of rat β_1-casein (Fig. 2a) and β_2-casein (Fig. 3a) clearly show the close similarity between these two proteins, with nearly all the corresponding peptides showing coincidental elution times and similar peak area ratios, the only notable

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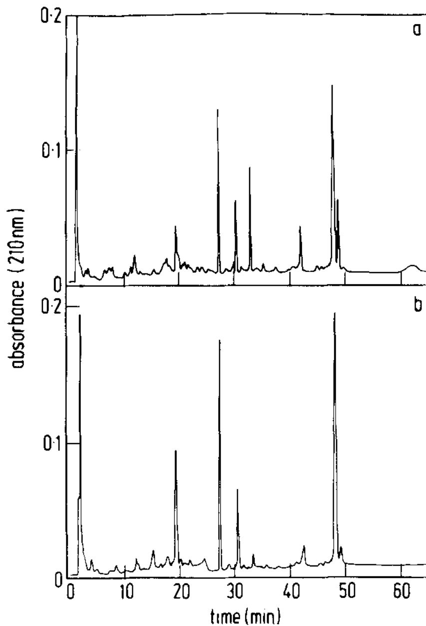

Figure 1.

Reversed phase HPLC analysis of the tryptic peptides of rat dephosphorylated α_1-casein (a) and rat phosphorylated α_1-casein (b) on a μBondapak Fatty Acid Analysis column (30cm x 3.9mm I.D.) using a linear 60min gradient from water-16mM orthophosphoric acid to 50% acetonitrile-50% water-16mM orthophosphoric acid at a flow rate of 2.0ml/min; temperature 18°, sample loading (150μg).

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exception being the peptide with t_R = 44.0min, which although present in both samples, was considerably reduced in peak area in the digest of the rat β_2-casein. Comparison of both these elution profiles with that of the 2h tryptic digest of the dephosphorylated rat α_1-casein (Fig. 1a) shows that there is little structural similarity between the α_1- and β-caseins.

As can also be seen by comparison of the phosphorylated and dephosphorylated rat caseins, these reversed phase HPLC procedures can also be used to obtain information on the

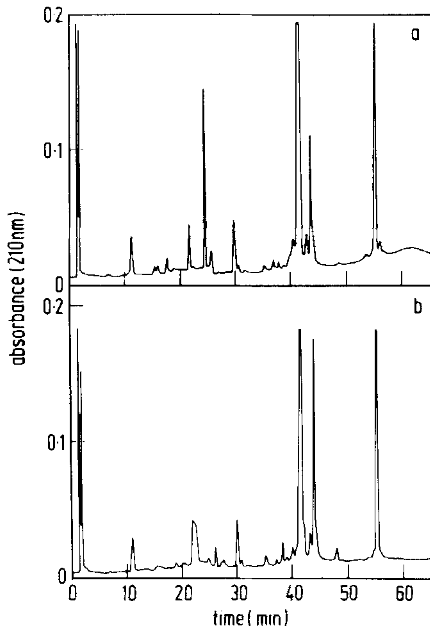

Figure 2.

Reversed phase HPLC analysis of the tryptic peptides of rat dephosphorylated β_1 casein (a) and rat phosphorylated β_1-casein (b). Chromatographic conditions as in Figure 1.

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distribution of phosphate groups within the native casein molecules. For example, comparison of the tryptic peptides obtained from the dephosphorylated and phosphorylated forms of rat β_1-casein (Fig. 2a and 2b respectively) reveals that the enzymatic removal by potato acid phosphatase of the phosphate groups affects the elution times of only several characteristic peptides, i.e. the disappearance of the peptides with t_R 22.5 and 48mins present in the elution profile of the phosphorylated rat β_1-casein and the appearance of a major peptide peak at t_R 24.5 and several minor peaks at t_R 15, 18 and 56min. Comparable analyses of rat β_2-casein show similar changes in the elution profiles. It is note-

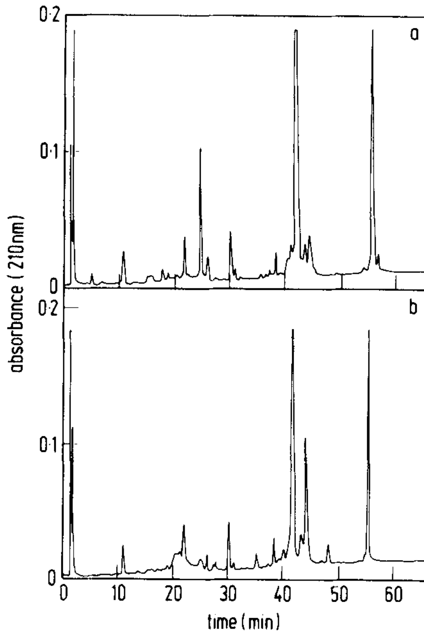

Figure 3.

Reversed phase HPLC analysis of the tryptic peptides of rat dephosphorylated β_2-casein (a) and rat phosphorylated β_2-casein (b). Chromatographic conditions as in Figure 1.

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worthy that comparison of the elution profiles of the tryptic digests of the phosphorylated β_1- and β_2-caseins also reveal a close similarity except for the region which elutes near to 20-24min. Other studies [2] have shown that rat β_1-casein contains six phosphate groups and rat β_2-casein seven phosphate groups per molecule. The essentially identical profiles for the tryptic maps of these two related caseins would be in accord with the small changes in polarity resulting from these phosphorylation patterns. Since only a few peptides were affected by dephosphorylation of the β-caseins the phosphate groups must be confined to restricted regions of the polypeptide chain, as is also found in the major bovine caseins [12]. Furthermore, most

of the peptides which do not contain phosphate groups elute late in the gradient, indicative of nonpolar peptides. This suggests that rat caseins contain large hydrophobic domains, a characteristic also shown by other mammalian caseins [12]. The similarity between the β-caseins contrasts the numerous changes observed in the elution profile of the tryptic digest of rat α_1-casein following dephosphorylation (Fig. 1a,b).

CONCLUSION

In conclusion, methods are reported for the enzymatic mapping by reversed phase HPLC of rat β_1- and β_2 caseins. These methods clearly confirm the close structural similarity of the rat β-caseins and their unrelatedness to rat α_1-casein. In addition these techniques permit an analysis of the phosphorylation patters of these caseins.

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