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Characterization of bovine κ-casein fractions and the kinetics of chymosin-induced macropeptide release from carbohydrate-free and carbohydrate-containing fractions determined by high-performance gel-permeation chromatography

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Bovine κ-casein was fractionated at pH 8.0 on DEAE-Sepharose with an NaCl gradient, followed by DEAE-cellulose chromatography using a decreasing pH gradient from pH 6.0 to 4.5. At least ten components could be identified, each differing in *N*-acetylneuraminic acid (NeuAc) and/or phosphorus content. Two components appeared to be multiply-phosphorylated, but did not contain NeuAc. The possible significance of this finding in relation to the mode of phosphorylation and glycosylation *in vivo* is discussed. A carbohydrate-free fraction as well as two NeuAc-containing fractions were compared in their substrate behaviour towards the action of the milk-clotting enzyme chymosin at pH 6.6 and 30 °C. To this end the trichloroacetic acid-soluble reaction products were analysed by high-performance gel-permeation chromatography. In order of increasing carbohydrate content the $k_{cat.}$ values found ranged from 40 to 25 s^{-1} and the K_m values from 9 to 3 μM; the overall substrate properties of these components as reflected by the kinetic parameter $k_{cat.}/K_m$ ranged from 5 to 8 $\mu M^{-1} \cdot s^{-1}$. Irreversible polymerization of the carbohydrate-free fraction brought about a more-than-2-fold increase in K_m, the $k_{cat.}$ value remaining virtually constant. The $k_{cat.}/K_m$ found for the cleavage of whole κ-casein at pH 6.6 was of the same magnitude as the $k_{cat.}/K_m$ found for the polymerized carbohydrate-free fraction (i.e. about 3 $\mu M^{-1} \cdot s^{-1}$). No indication of substrate inhibition was found for the carbohydrate-free fraction.

INTRODUCTION

Although considerable quantities of bovine milk are clotted daily by enzymes in industry and on farms, the kinetic parameters of even the most prominent reaction, the action of the enzyme chymosin (EC 3.4.23.4) on its substrate κ-casein, have not been established definitively, in spite of numerous studies on the subject going back for about 30 years, which yielded conflicting results (Wheelock *et al.*, 1972; Dalgleish, 1982). It is not surprising that the catalytic-centre activities ($k_{cat.}$) given in these studies are difficult to compare, since only the weight of the enzyme and not its activity was used to calculate $V_{max.}$. But values for K_m, which should in principle be much less dependent on enzyme activity, are reported in the range of 1–500 μM (Dalgleish, 1982). Lack of a suitable method to determine the amount of product formed during the enzymic reaction has certainly contributed heavily towards the confusion. A quick and sensitive method, such as h.p.l.c., seems to be ideal to apply to this problem.

We used high-performance gel-permeation chromatography (h.p.g.p.c.) to determine the trichloroacetic acid-soluble κ-casein macropeptide (i.e. fragment 106–169), which is released from κ-casein after specific cleavage of the Phe^{105}–Met^{106} peptide linkage by chymosin. This procedure has recently been described by Van Hooydonk & Olieman (1982). The situation, however, is complicated by at least two factors. First, κ-casein exists in milk predominantly on the surface of the casein micelles, particles with a broad size distribution and a weight-average diameter of 190 nm (Horne, 1984). In solutions of isolated κ-casein, mainly particles with an M_r of 600000 and a diameter of 23 nm are present, whereas under certain conditions, at low concentrations (< 25 μM), only monomers (M_r 19000) are encountered. The widely differing diffusion coefficient of the substrate in its various forms could influence the kinetics of the reactions, as has been proposed by Addeo *et al.* (1984) and Van Hooydonk *et al.* (1984). By performing experiments with κ-casein particles of different sizes we have tried to assess the importance of this effect.

The second factor is the heterogeneity of bovine κ-casein. In addition to the amino-acid replacements in the two genetic variants that are known at present, each peptide chain may differ by post-translational modifications in which various amounts of phosphate and carbohydrate are attached, the latter in the form of tri- and tetra-saccharide units of *N*-acetylneuraminic acid (NeuAc), hexose (usually galactose, Gal) and *N*-acetylhexosamine (usually GalNAc), e.g. NeuAcα2-3Galβ1-3GalNAcβ1-Thr/Ser) or Galβ1-3(NeuAcα2-6)GalNAcβ1-Thr/Ser or NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAcβ1-Thr/Ser (Van Halbeek *et al.*, 1980). The carbohydrate units are *O*-glycosidically linked to threonine or serine residues in the region 127–141 of the peptide chain (Eigel *et al.*, 1984). All κ-casein molecules contain at least one ester phosphate group. About 40% of the κ-casein is not glycosylated, and this component is the slowest moving band on electrophoresis in an alkaline starch gel (Fig. 1). It is conceivable that the modifications which occur in the region of 127–141 of the

Abbreviations used: NeuAc, *N*-acetylneuraminic acid; Nle, norleucine; -OMe, methyl ester; PEG, poly(ethylene glycol); h.p.g.p.c., high-performance gel-permeation chromatography; GalNAc, *N*-acetylgalactosamine.

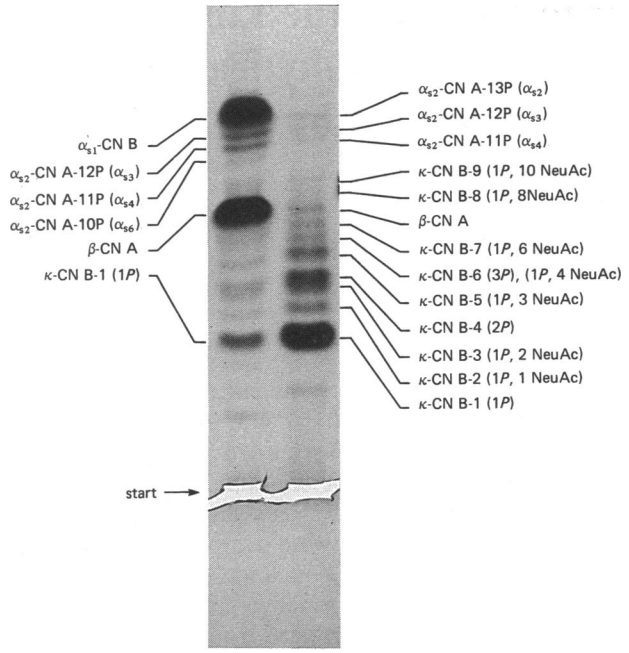


Fig. 1. Starch-gel pattern of whole casein (left) and whole κ-casein, both prepared from milk from a cow which was homozygous for the κ-CN B variant

Conditions were as follows: pH 8.6, 5 M-urea, 0.03 M-mercaptoethanol and 76 mM-Tris/citrate buffer. The number of phosphate (*P*) and NeuAc groups for each κ-casein component is given in parentheses. Also given in parentheses are the traditional names for the α_{s2}-CN components.

primary structure influence the susceptibility of the Phe^{105}–Met^{106} bond towards enzymic attack. Most workers (Addeo *et al.*, 1984; Doi *et al.*, 1979*a*; Van Hooydonk *et al.*, 1984; Sinkinson & Wheelock, 1970) find that the rate of κ-casein macropeptide release decreases with increasing carbohydrate content, whereas Chaplin & Green (1980) do not observe an effect at all. We have tried to settle this point by a study of the kinetics of chymosin action on three well-defined κ-casein fractions, two of which contain NeuAc. For comparison, we have also determined the kinetics of the splitting of whole κ-casein. The conditions of pH and ionic strength chosen for the kinetic experiments were close to those present in milk. Further, the kinetic data found for the carbohydrate-free κ-casein are compared to those reported previously by Visser *et al.* (1976, 1977, 1980) for a series of peptide substrates having an amino acid sequence identical with that around the Phe^{105}–Met^{106} bond of κ-casein.

The κ-casein fractions which were used in the kinetic experiments were selected from those obtained from whole κ-casein by using an improved (double) fractionation procedure on DEAE-cellulose and DEAE-Sepharose columns. By this procedure the electrophoretically faster-moving fractions could now be separated, whereas formerly (Vreeman *et al.*, 1977) only the first four components could be isolated in a sufficiently pure state. As considerable confusion exists about the structure of the faster-moving components (Eigel *et al.*, 1984), these fractions were analysed to obtain an improved description of the composition of whole κ-casein from a cow homozygous for the B-variant.

MATERIALS AND METHODS

Isolation and purification of κ-casein fractions

Whole κ-casein was prepared by the McKenzie & Wake (1961) procedure. Other workers fractionated whole κ-casein on a DEAE-cellulose column at pH 7.0 with an NaCl gradient (Mercier *et al.*, 1968; Doi *et al.*, 1979*b*). Since at that pH the κ-casein phosphate groups are not fully charged, their p*K* being about 6.5, we decided to fractionate first at pH 8.0 on DEAE-Sepharose with an NaCl gradient. The fractions thus obtained were subsequently purified on DEAE-cellulose with a pH gradient as described previously (Vreeman *et al.*, 1977). The pH started at 6.0 (6.5 for the A-variant) and decreased to 4.5. The fractions that contain extra phosphorus instead of NeuAc (same net charge at pH 8.0) will thus be washed from the DEAE-cellulose column first because of their diminished charge at the lower pH in the pH-gradient experiment. DEAE-cellulose was Cellex-D (standard capacity) from Bio-Rad and DEAE-Sepharose CL-6B from Pharmacia.

Starch-gel electrophoresis and phosphorus determinations were performed as described by Schmidt (1964) and Griswold *et al.* (1951) respectively. NeuAc was liberated by neuraminidase from *Vibrio comma* (*cholerae*) (Behringwerke A.G., Marburg, Germany) and determined by the thiobarbituric acid method of Warren (1959).

For the nomenclature of κ-casein components the guidelines of the American Dairy Science Association Committee (Eigel *et al.*, 1984) are followed.

Substrate and enzyme solutions

Substrate stock solutions (about 50 μM) were prepared in potassium phosphate buffer, *I* 0.05, pH 6.6 (a solution of 1.01 g of KH_2PO_4 in distilled water, which was titrated with 3 M-KOH and adjusted to 250 ml). The carbohydrate-free κ-CN B-1 component exists in solution in different stable states of aggregation, depending on treatment of the protein solution. If dissolved and kept at temperatures below 15 °C, the κ-CN B-1 system is in a reversible monomer $\rightleftharpoons$ polymer equilibrium. In such a system, below the critical micelle concentration, only monomers exist. Above that concentration there exists a mixture of monomers and polymers (micelles). The polymer has an M_r of about 600000 and the critical micelle concentration is 25 μM (Vreeman *et al.*, 1981).

On heating κ-CN B-1 solutions at 35 °C for 5 h the system becomes irreversible, i.e. the κ-casein polymers do not fall apart into monomers any more when diluted below 25 μM and their M_r remains at 600000 (H. J. Vreeman, unpublished work). The sedimentation coefficient ($s_{20,w}$) of these irreversible polymers is 12 S, about the same as that which was found for the reversible particle.

On heating carbohydrate-free κ-casein in solution at 35 °C for 8 h, particles of increased size, with a sedimentation coefficient of 22 S, are formed. To obtain 50 μM stock solutions of irreversibly polymerized κ-CN B-1 casein, a 50 μM and a 250 μM solution of κ-CN B-1 casein were kept at 35 °C for 5 h and for 8 h respectively, the latter being subsequently diluted to 50 μM. The exact concentration in these stock solutions was determined by

spectrophotometric assay at 291 nm after 4-fold dilution with 0.1 M-NaOH. The resulting concentration was based on an M_r of 19030 for the substrate's peptide chain and taking $A_{291}^{10\%}{}_{1\,cm} = 13.11$ under alkaline conditions (Vreeman *et al.*, 1977). For kinetic measurements the substrate stock solutions were diluted to 3–45 μM with the above phosphate buffer.

Chymosin (rennin, EC 3.4.23.4) was a purified preparation (De Koning, 1968) with a milk-clotting activity of 6.5×10^6 Soxhlet units (Foltmann, 1971). Its proteolytic activity amounted to 540 ± 30 mkat/kg as determined spectrophotometrically at 230 nm and 30 °C by using the synthetic hexapeptide Leu-Ser-Phe-Nle-Ala-Ile-OMe (1 mM in 0.05 M-sodium acetate, pH 4.7) as the substrate (Visser & Rollema, 1986). A stock solution (0.13 mg/ml corresponding to 4.2 μM when the M_r is taken to be 30000 (Foltmann, 1971) was made in 0.05 M-sodium acetate/1 M-NaCl buffer, pH 5.2 [although the M_r has now been established from the amino acid sequence as being 35652 (Foltmann *et al.*, 1977), we have used 30000 in order to facilitate the comparison of $k_{cat.}$ values with those found previously (Visser *et al.*, 1976, 1977, 1980) for the cleavage of κ-casein analogues]. For further dilution, precautions were necessary to avoid adsorption of the enzyme at an interface (Friedenthal & Visser, 1985). Dilutions were made with the above enzyme buffer, pH 5.2, in which 0.1% PEG 20000 had been included; vials in which the dilutions were made were pre-rinsed with the PEG-containing buffer and dried before use. To reach equilibrium conditions, the diluted enzyme solutions were kept at 0 °C for at least several hours before their use in the kinetic experiments; immediately after dilution and again just before use the proteolytic activities were measured spectrophotometrically as described above. During the subsequent series of incubations, a 'fresh' batch of diluted enzyme solution (continuously kept at 0 °C) was taken from time to time to avoid possible contamination caused by repeated sampling. Therefore, small decreases in proteolytic activity were corrected for by taking the original activity (i.e. 540 mkat/kg) as the 100% reference.

Effect of trichloroacetic acid on the solubility of κ-casein fractions and of the corresponding macropeptides

To 250 μl of a 64 μM-κ-casein solution in pH 6.6 buffer, 5 μl of a 20-fold diluted enzyme stock solution was added. After incubation at 30 °C for 3 h (during which time 100% cleavage of the substrate was achieved), 85 μl of trichloroacetic acid solution of an appropriate concentration was added so as to attain a final trichloroacetic acid concentration in the range of 1–16% (w/v); the macropeptide concentration in this mixture was 47 μM. After the mixture had been left at room temperature for 25 min, the precipitate formed was removed by centrifugation at 5000 *g*. In the supernatant the macropeptide content was determined by h.p.g.p.c. as described below. Since the content at a low trichloroacetic acid concentration (1–2%) was always constant, this value was considered as 100% recovery (i.e. 47 μM).

For the determination of the κ-casein solubility as a function of the final trichloroacetic acid concentration the same procedure was followed for the sample preparation, except that 5 μl of enzyme buffer (instead of enzyme solution) were added to the appropriate substrate solution. After trichloroacetic acid precipitation the κ-casein concentration in the supernatant was established spectrophotometrically under alkaline conditions at 291 nm (Vreeman *et al.*, 1977) with proper corrections for blank solutions.

The enzyme–substrate reaction

To 250 μl of substrate solution (3–45 μM), pre-equilibrated at 30 °C, 5 μl of a 40-fold-diluted enzyme stock solution was added (the final enzyme concentration was about 2 nM). After incubation at 30 °C for 60 s the reaction was stopped by vigorously vortex-mixing into the sample 85 μl of a 12, a 28 or a 48% trichloroacetic acid solution, resulting in final trichloroacetic acid concentrations of 3, 7 and 12% (w/v) in the case of κ-CN B-1, κ-CN B-5 and κ-CN B-7 respectively. In the incubation with whole κ-CN B and polymerized κ-CN B-1, final trichloroacetic acid concentrations of 3% were used. After being left at room temperature for 25 min, the precipitates were removed by centrifugation at 5000 *g* and the supernatants subjected to h.p.g.p.c. analysis in duplicate runs.

H.p.g.p.c. analysis

The analytical procedure was essentially that described by Van Hooydonk & Olieman (1982), with some modifications. The analyses were performed by using a system consisting of a M6000 A pump (Waters Associates), an ISS-100 automatic sample injector (Perkin–Elmer), a 2.3 cm × 0.3 cm i.d. (internal diameter) guard column filled with I-125 packing material (37–53 μm particle size; Waters Associates), two coupled TSK 2000 SW columns (30 cm × 0.75 cm i.d.; Varian) maintained at 35 °C in a Kipp Analytica 9222 column oven, a model-450 variable-wavelength u.v. detector (Waters Associates), a BD-9 two-channel recorder (Kipp) and a Spectra Physics model SP4100 integrator. For saturation of the mobile phase with solubilized silica material, a column (30 cm × 0.46 cm i.d.) filled with silica gel (0.2–0.5 mm particle size; Merck) was placed between the buffer pump and the sample injector.

The eluent consisted of a solution of 1.74 g of K_2HPO_4, 12.37 g of KH_2PO_4 and 21.41 g of Na_2SO_4, adjusted to 1000 ml with distilled water (pH 5.6). To prevent bacterial growth and to keep the eluent degassed, it was filtered through a 0.45 μm-pore-size filter (Millipore), kept at 90 °C and stirred continuously with a magnetic bar.

It was important to avoid unwanted adsorption of peptide material to the guard and separation columns. This was effected by successive injections with a fixed amount of macropeptide (obtained by complete cleavage of a 3 μM solution of the appropriate κ-casein fractions with chymosin and precipitating the protein material with trichloroacetic acid) until a constant response was observed; this conditioning procedure was repeated before each new set of analytical runs.

For each analysis, 50 μl of the sample (i.e. the supernatant after trichloroacetic acid precipitation) was injected automatically. The flow rate was adjusted to 1.0 ml·min^{-1} and the u.v. detector operated at 205 nm and 0.1 *A* unit full scale. In a series of analytical runs the period between two injections was 23 min.

Peak areas for the macropeptide were converted into concentrations by comparison with peak areas representing known amounts of macropeptide obtained after

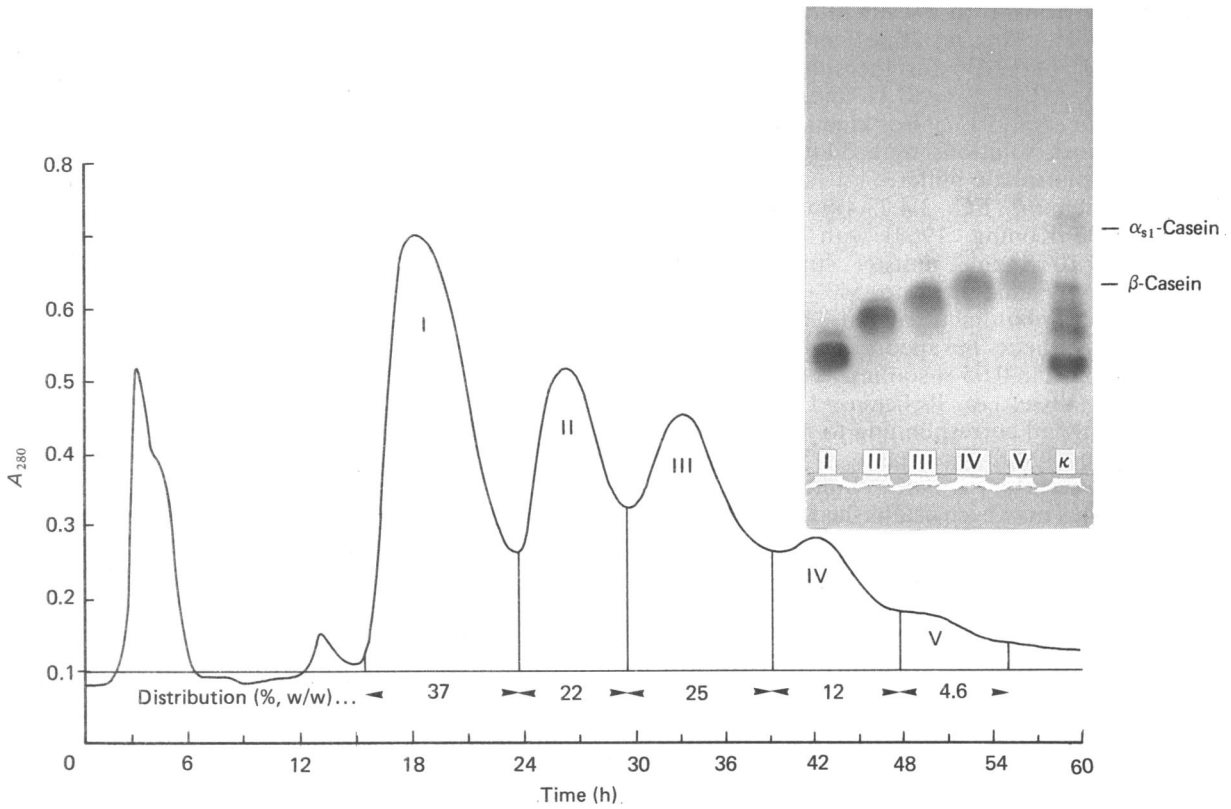


Fig. 2. Fractionation of κ-CN B on DEAE-Sepharose CL-6B

Conditions were as follows: 20 cm × 50 mm column; temperature 25 °C; solvent 5 M-urea/0.02 M-Tris/HCl/1 mM-glycylglycine/1 mM-mercaptoethanol, pH 8.0; protein applied, 5 g. The gradient was solvent plus 40–200 mM-NaCl in 48 h; the flow rate was 80 ml/h; the gradient was started at 6 h on the abscissa. The weight percentages of the fractions are indicated along the abscissa. The inset shows a starch-gel pattern of fractions I–V; the reference is whole κ-casein B. The fraction between 0 and 6 h contains only a small amount of protein.

complete conversion of the κ-casein component in question. Before trichloroacetic acid precipitation the incubation mixtures prepared for this calibration were heated at 80 °C for 10 min to inactivate chymosin, and various concentrations of the corresponding κ-casein fraction were added to simulate and correct for losses of macropeptide due to co-precipitation with κ-casein.

Calculation of kinetic parameters

Initial rates of macropeptide production as derived from the h.p.g.p.c. analyses were used to construct plots of v against [S], [S]/v against [S] (Hanes, 1932) and $1/v$ against 1/[S] (Lineweaver & Burk, 1934) using a BASIC program on a Hewlett–Packard calculator, model 9845. From the linear plots, which were weighted for inversion (Dowd & Riggs, 1965), the kinetic parameters K_m, $k_{cat.}$ and $k_{cat.}/K_m$ were calculated; a correction was made for the extent of hydrolytic cleavage during the enzymic reaction (Visser *et al.*, 1976).

RESULTS

κ-Casein fractionation

Five distinct fractions were eluted from the DEAE-Sepharose column at pH 8.0 with an NaCl gradient. A typical chromatogram is given in Fig. 2. The fractions were run on a starch gel at pH 8.6 and, as can be seen from the gel pattern in Fig. 2, the fourth fraction moves almost as fast as β-casein, whereas the fifth fraction moves even faster. Roman numerals were given to these fractions to distinguish them from those obtained from them later on. The recovery based on total yield is also given in Fig. 2 (bottom).

The properties of fractions I and II, which have been analysed previously (Vreeman *et al.*, 1977) are included in Table 1. Band κ-CN B-2 is probably a mixture of κ-casein having one NeuAc group and κ-casein modified in another way (e.g. deamidation).

Each of the fractions III, IV and V proved to be very heterogeneous on re-chromatography with a pH gradient (cf. Fig. 3). The higher the electrophoretic mobility of the parent fraction the more heterogeneous the fraction appeared to be.

About 70% of fraction III consisted of band κ-CN B-5 (cf. Table 1). About 50% of subfractions III-0, III-1 and III-4 consisted of κ-CN B-5.

The results for fractions IV and V are more complex than those for fraction III. The main component in IV (IV-4 and IV-5, together 5.6%) has one phosphate and six NeuAc groups. It is noteworthy that the average NeuAc content of IV is 7.15% (corresponding to an average of five NeuAc groups per molecule), whereas the fractions which contribute to this average range from no, up to seven, NeuAc groups per molecule. The fraction without NeuAc (i.e. IV-1) contains two extra phosphate groups. The same component is the main constituent of

Table 1. Composition of fractions III, IV and V

NeuAc and phosphorus content of subfractions III, IV and V obtained by pH-gradient chromatography (cf. Fig. 3). The data for I and II are inferred from previous work (Vreeman *et al.*, 1977).

Subfraction	(%, Proportion w/w) of starting κ-CN B	NeuAc		P		Nomenclature (cf. Fig. 1)
		(%, w/w)	(mol/mol)	(%, w/w)	(mol/mol)	
(I)	34	–	0	–	1	κ-CN B-1
	3	–	1	–	1	κ-CN B-2
(II)	11	–	2	–	1	κ-CN B-3
	11	–	0	–	2	κ-CN B-4
III-0*	7.4	3.32	†	0.20	†	κ-CN B-4+B-5
III-1	1.5	4.00	†	0.20	†	κ-CN B-4+B-5
III-2	5.0	4.75	3	0.13	1	κ-CN B-5
III-3	4.1	4.76	3	0.14	1	κ-CN B-5
III-4	7.0	4.51	†	0.15	1	κ-CN B-5+B-6
IV-1	1.0	0.63	0	0.37	3	κ-CN B-6 (3 *P*)
IV-2	1.7	4.35	4	0.20‡	1	κ-CN B-6 (4 NeuAc)
IV-3	1.8	6.68	†	0.19	†	κ-CN B-6+B-7
IV-4	3.5	8.26	6	0.12	1	κ-CN B-7
IV-5	2.1	8.75	6	0.13	1	κ-CN B-7
IV-6§	1.9	9.36	7	0.13	1	κ-CN B-7+B-8
V-1	0.23	0.0	0	0.35	3	κ-CN B-6 (3 *P*)
V-2†	0.98	–		–		κ-CN B-6+B-7
V-3†	0.94	–		–		κ-CN B-6+B-7
V-4	0.87	8.40	6	0.19‡	1	κ-CN B-6+B-7
V-5	0.76	10.00	8	0.14	1	κ-CN B-8
V-6§	0.82	11.65	9	0.13	1	κ-CN B-8+B-9

* Subfraction III-0 (not shown in Fig. 3) was eluted during the prewash.
† Fraction consists of multiple bands, therefore no values given or determined.
‡ P content somewhat too high for 1 mol/mol, probably due to contamination by preceding fraction.
§ Broad and fuzzy band, consists possibly of two or more components.

V-1. A combination of extra phosphate groups together with NeuAc groups in one fraction was not found.

Gel patterns for a selection of the fractions are shown in Fig. 4: fraction III-3 (κ-CN B-5), fraction IV-4 (κ-CN B-7), V-5 (κ-CN B-8) and IV-1 [κ-CN B-6 (three phosphate groups)].

To check whether these fractions, especially the ones around the β-casein position on a starch gel, could be classified as κ-casein, samples of all the fractions from IV and V were incubated with chymosin and run on a starch gel. In all cases the casein band disappeared and a band in the *para*-κ-casein (fragment 1–105) position appeared on the gel (result not shown). This confirms that all electrophoretically distinguishable post-translational modifications are in the macropeptide part of the κ-casein molecule.

From these results the number of phosphate- and NeuAc-groups per molecule were assigned to the κ-casein bands in Fig. 1. The fraction with an average of nine NeuAc groups appears broad and fuzzy on a starch gel and could be a mixture of components with eight and ten NeuAc groups. In that case κ-CN B-9 has ten NeuAc-groups. If the fraction is homogeneous, κ-CN B-9 has nine NeuAc-groups. Note that κ-CN B-6 consists of two components, both having four more negative charges at pH 8.6 than does κ-CN B-1.

Effect of trichloroacetic acid concentration on the solubility of κ-casein fractions and of the corresponding macropeptides

The heterogeneity of κ-casein, attributable to a variable carbohydrate and/or phosphate content, is reflected in the different solubilities of its fractions (such as κ-CN B-1, κ-CN B-5 and κ-CN B-7) in trichloroacetic acid. As the substituents are exclusively attached to the macropeptide part of κ-casein, the solubility of the macropeptide is also strongly influenced by the trichloroacetic acid concentration. The non-variable cleavage product, *para*-κ-casein, appears to be definitely insoluble, even at low trichloroacetic acid concentration. Fig. 5 shows the recovery of κ-CN B-1, κ-CN B-5 and κ-CN B-7 as well as that of their corresponding macropeptides at room temperature as a function of the final trichloroacetic acid concentration. It appeared that the presence of residual κ-casein or *para*-κ-casein in the sample for h.p.g.p.c. can easily disturb the analysis and therefore the results of the kinetic experiment. So the optimum trichloroacetic acid concentration to be used should be the one at which maximal protein (κ-casein and *para*-κ-casein) and minimal macropeptide precipitation occurs. From Fig. 5 it is seen that this condition is fulfilled by taking 3, 7 and 12% trichloroacetic acid in the experiments with κ-CN B-1, κ-CN B-5 and κ-CN B-7 respectively. In the experiment with whole κ-casein a trichloroacetic acid concentration of 3% was sufficient to precipitate the protein completely and to keep the macropeptide in solution.

Determination of kinetics

In Fig. 6 a typical h.p.g.p.c. elution pattern of three identical κ-CN B-1 macropeptide samples (injected during the final stage of column conditioning) is depicted. The macropeptide peak appears sufficiently

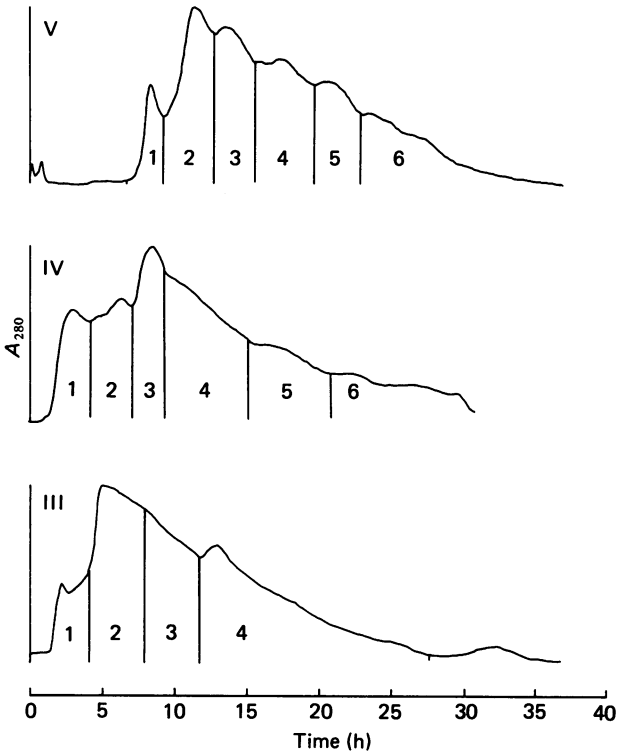


Fig. 3. Fractionation of fractions III, IV and V by pH-gradient chromatography

Conditions were as follows: column length, 20 cm; diameter, 16 mm; material, Cellex-D; the flow rate was 22.8 ml/h and the eluate was collected at a rate of three tubes/h. After the protein had been adsorbed to the column the adsorption buffer was allowed to wash the column for 3.5 h. After this prewash, the gradient was started (0 h on the abscissa). III, Adsorption at pH 5.8, gradient pH 5.8–4.5 + 50 mM-NaCl in 48 h. Subfraction III-0 (not shown) was eluted during the prewash. IV, Adsorption at pH 6.0, gradient pH 5.8–4.5 + 50 mM-NaCl in 48 h. V, Adsorption at pH 6.0, gradient pH 5.5–4.0 + 50 mM-NaCl in 48 h.

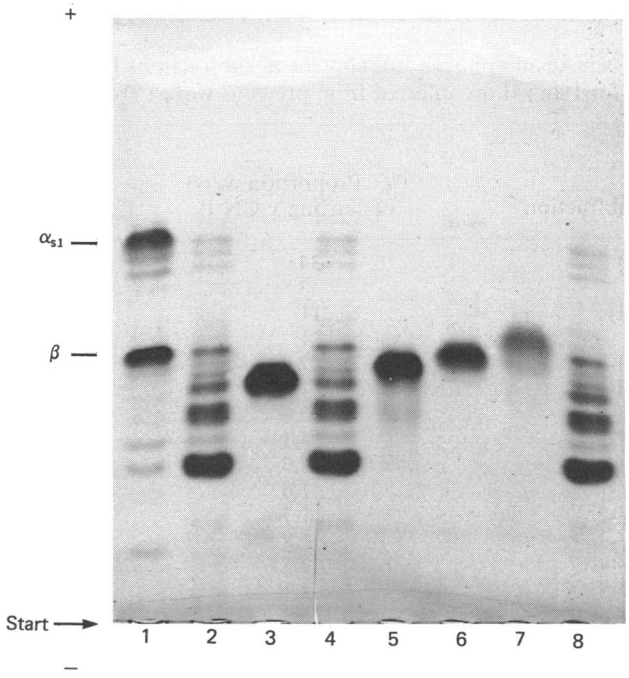


Fig. 4. Starch-gel pattern of purified fractions of κ-casein B

Lane 1, whole casein; lane 2, κ-CN B; lane 3, fraction III-3 (κ-CN B-5); lane 4, κ-CN B; lane 5, fraction IV-1 (κ-CN B-6 3P); lane 6, fraction IV-4 (κ-CN B-7); lane 7, fraction V-5 (κ-CN B-8); lane 8, κ-CN B.

separated from the other peaks (mainly originating from trichloroacetic acid) to be accurately quantified.

The v-against-[S] and $1/v$-against-1/[S] plots for the cleavage of κ-CN B-1, κ-CN B-5 and κ-CN B-7 are shown in Figs. 7(*a*) and 7(*b*). In one experiment (results not shown) the kinetics of the κ-CN B-1 cleavage were monitored further to much higher substrate concentrations. An almost constant $V_{max.}$ value was observed up to at least 400 μM, indicating that substrate inhibition was absent.

In Table 2 the kinetic parameters for the cleavage of κ-CN B-1, κ-CN B-5 and κ-CN B-7, as well as of polymerized κ-CN B-1 and κ-CN B, are compared. It is seen that $k_{cat.}$ (and thus $V_{max.}$) slightly decreases with increasing NeuAc content of the κ-casein component. On the other hand one observes a more or less reverse effect for K_m, with a somewhat larger difference between κ-CN B-1 and κ-CN B-5 than between κ-CN B-5 and κ-CN B-7. Consequently, the overall substrate properties, as reflected by the parameter $k_{cat.}/K_m$, show an optimum for the κ-CN B-5 substrate, but altogether the differences are not greater than a factor of about 1.7.

When comparing the kinetics for the (mainly) monomer and polymerized forms of the κ-CN B-1 substrate, one sees a marked decrease in $k_{cat.}/K_m$ for the latter, which is predominantly caused by a higher K_m. Furthermore, it is worth noting that the $k_{cat.}/K_m$ value for polymerized κ-CN B-1 is close to the one found for whole κ-casein.

DISCUSSION

κ-Casein fractionation and classification of components

Comparison of our initial fractionation at pH 8.0 with that of others at pH 7.0 (Doi *et al.*, 1979*a*; Mercier *et al.*, 1968), all performed with an NaCl gradient, shows that, at pH 8.0, five fractions are obtained as against seven at pH 7.0. The fifth fraction at pH 8.0, which has a starch-gel mobility higher than that of β-casein, was not recovered by other workers, probably because this fraction contains only 5% of κ-CN B.

The seven fractions obtained at pH 7.0 encompass the bands κ-CN B-1–κ-CN B-7, as can be seen from the work of Mercier *et al.* (1968). Apparently κ-CN B-2 was obtained in those studies as a small separate fraction (at pH 7.0), whereas this band is not separated from κ-CN B-1 under our conditions at pH 8.0. This leaves two additional small fractions at pH 7.0 [fractions 4 and 6 of Mercier *et al.* (1968)] which are not separated at pH 8.0 from fractions II, III and IV.

In itself this could be an indication that part of κ-casein differs from the rest in having a group with a pK between 6 and 8. Candidates for this would be N-terminal NH_2, histidine and ester phosphate.

The results of Doi *et al.* (1979*a*) confirm those of

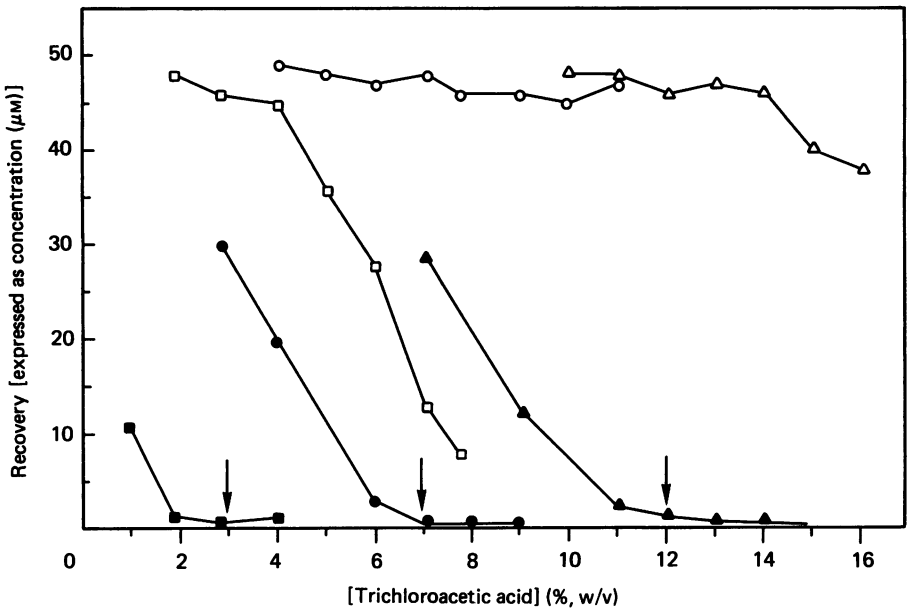
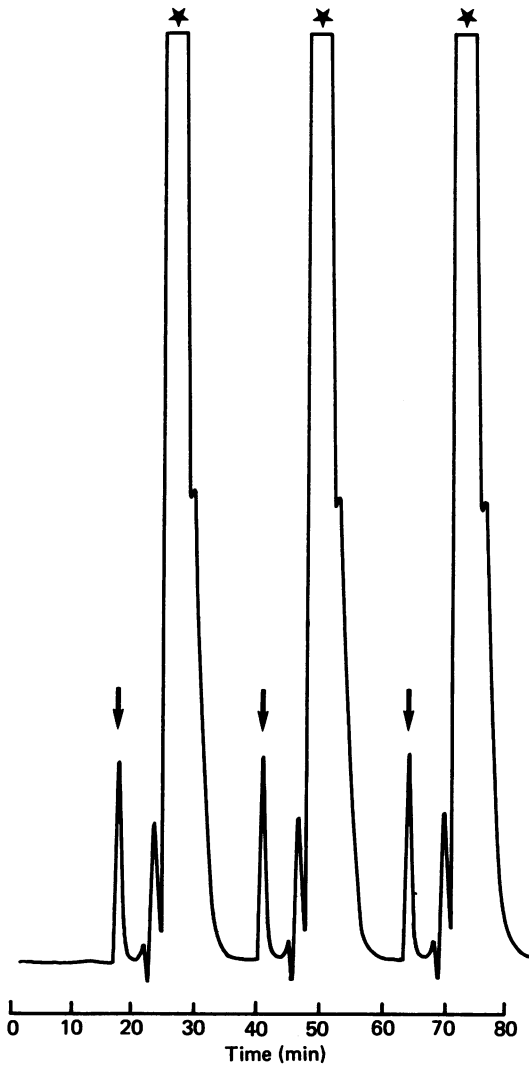


Fig. 5. Recovery of soluble κ-CN B-1, κ-CN B-5 and κ-CN B-7 and their respective macropeptide parts as a function of the trichloroacetic acid concentration used in the protein-precipitation step

■ = κ-CN B-1 casein; ● = κ-CN B-5 casein; ▲ = κ-CN B-7 casein. The open symbols represent the corresponding macropeptides. Starting concentrations were 47 μM. For further experimental details, see the text.



Mercier *et al.* (1968). The former's fractions P-X and P-Y (equivalent to the latter's 4 and 6) have a slightly higher ester-phosphorus content than do the other fractions which they isolated. This would argue for the case that these fractions contain the two components which we have identified as having one and two extra phosphate groups and no NeuAc. The inconsistency is that the two peaks are eluted from the column at pH 7.0 after the accompanying main peaks instead of in front of these peaks.

Of the two components mentioned above, the one with a total of two ester phosphate groups has already been described previously (Vreeman *et al.*, 1977) as κ-CN B-4. It was found subsequently that its second phosphate group was linked to Ser-127 (Mercier, 1981). The second component is κ-CN B-6, which has a total of three phosphate groups and no NeuAc. This fraction has the same mobility on a starch gel as has the fraction containing one phosphate and four NeuAc groups per molecule.

From a quantitative point of view, component κ-CN B-5 (one phosphate and three NeuAc groups) is the most important one of the fraction III, IV and V (cf. Table 1). The components κ-CN B-1–κ-CN B-5 contribute to about 80% of whole κ-casein.

In general, components with an uneven number of NeuAc groups seem to be quantitatively minor, with the notable exception of κ-CN B-5, which has three NeuAc groups. A fraction with five NeuAc groups was not found, and the fraction with seven NeuAc groups is very

Fig. 6. A typical h.p.g.p.c. elution pattern of three identical κ-CN B-1 macropeptide samples (injected during the final stage of column conditioning)

Macropeptide peaks are indicated by arrows; ✱, trichloroacetic acid. Detection was at 205 nm, 0.1 a.u.f.s. (absorbance unit full scale).

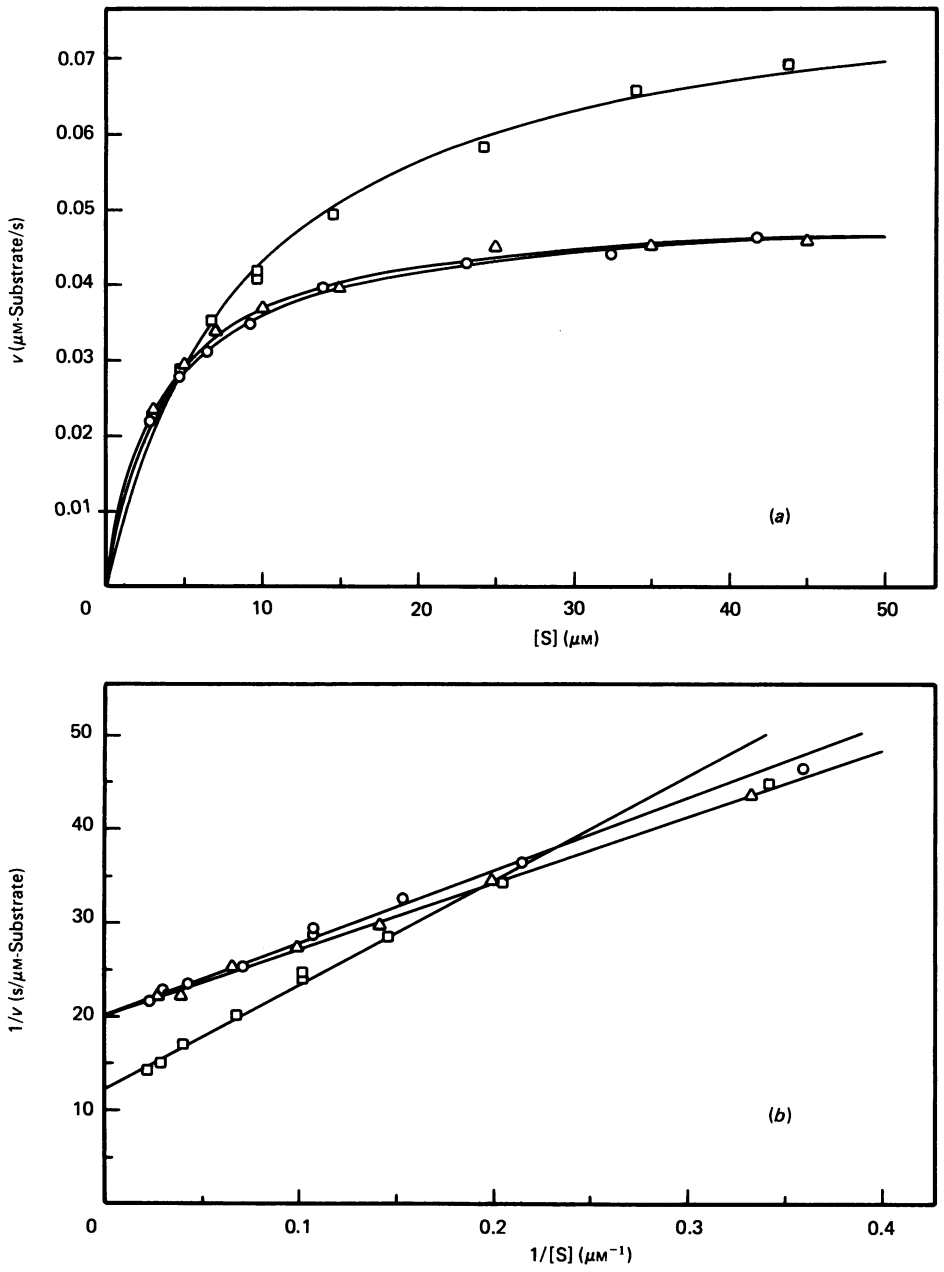


Fig. 7. Plots for the cleavage of κ-CN B-1 (□), κ-CN B-5 (○) and κ-CN B-7 (△) casein by chymosin at pH 6.6 and 30 °C

(*a*) Velocity-versus-substrate concentration plot; (*b*) Lineweaver-Burk plot ($1/v$ against 1/[S]). The enzyme concentrations used were 1.9, 1.6 and 2.0 nM respectively.

minor, as is the fraction with one NeuAc group (Vreeman *et al.*, 1977).

Ten NeuAc groups per molecule of κ-casein seems to be the maximum value, as we have no evidence of a component more heavily modified in this respect. This is borne out by Zevaco & Ribadeau-Dumas (1984), who found that four threonine residues (positions 131, 133, 135 and 142) and one serine residue (position 141) can serve as point of attachment for *N*-acetylgalactosamine (GalNAc), which is the terminal residue of the tri- and tetra-saccharide units mentioned in the Introduction. Consequently a maximum of ten NeuAc groups can be accommodated.

A total of nine different fractions for whole κ-casein was reported by Takeuchi *et al.* (1984), which is in good agreement with the number of bands in Fig. 1.

Mode of glycosylation and phosphorylation *in vivo*

Concerning the mechanism of modification by glycosylation and phosphorylation the following remarks can be made.

An average of about one GalNAc group and one phosphate group per peptide chain is found (Doi *et al.*, 1979*b*). The GalNAc group has a choice of five different positions on the κ-casein molecule. If one assumes that these sites are equivalent, i.e. the probability of attachment of a GalNAc group to the site does not depend on the position of the site in the primary

Table 2. Kinetic parameters of chymosin action on isolated whole κ-casein, κ-casein fractions and tryptic κ-casein fragments

The temperature was 30 °C unless otherwise indicated.

Substrate	pH	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($\mu M^{-1} \cdot s^{-1}$)	Reference
κ-CN B-7	6.6	24.9	3.5	7.1	*
κ-CN B-5	6.6	30.9	3.8	8.1	*
κ-CN B-1	6.6	43.4	8.9	4.9	*
κ-CN B-1 (12 S)	6.6	35.5	11.8	3.0	*
κ-CN B-1 (22 S)	6.6	59.9	21.5	2.8	*
κ-CN B	6.6	93.3	31.8	2.9	*
κ-CN B-1	6.2	68.9	40	1.4	†
κ-CN B	6.6	–	–	2	‡
κ-CN B	6.9	58–107	100	0.58–1.07	§
κ-CN B	6.4	–	1	–	‖
κ-CN B	6.95	–	66	–	¶
κ-CN B (f98–111)	6.6	46	29	1.6	‡
κ-CN B (f98–112)	6.6	48	23	2.1	‡

* Present paper.
† Carles & Ribadeau-Dumas (1985).
‡ Visser *et al.* (1980).
§ Garnier *et al.* (1968) from experiments carried out at various temperatures (25, 35 and 40 °C).
‖ Sanner & Kovács-Proszt (1973).
¶ Azuma *et al.* (1984); experiments performed at 37 °C.

structure, one can calculate the distribution of the GalNAc groups over the κ-casein molecule population, since the modification then occurs at random. The distribution for such a process is the Poisson distribution. The essential property of a random modification is that a certain percentage of the molecules will not be modified at all. This must be compensated by multiple modifications of other molecules if the average number of groups attached is one. For such an average one can predict that 37% will not be modified, 37% once modified, 18% twice, 6% three times and 2% four times modified. This can be compared with the results obtained here (Fig. 2 and Table 1) and by others (Mercier *et al.*, 1968). The carbohydrate-free fraction is about 34%, the sum of the fractions with one and two NeuAc groups (i.e. one GalNAc): 30%, the fractions with three and four NeuAc groups: 18%, with five and six NeuAc groups: 6%. This agreement between calculated and experimental results could suggest that glycosylation of cow κ-casein occurs by a random mechanism and that therefore the glycosylation sites are approximately equivalent and accessible.

Thus it would not be necessary to postulate a hidden position of part of the κ-casein inside the casein micelle, inaccessible to the glycosylating system, to explain the carbohydrate distribution (Slattery, 1978).

The average glycosylation level of κ-casein of other species can be much higher than that of cow κ-casein; for instance, about ten GalNAc groups per peptide chain are found in human (Yamauchi *et al.*, 1981; Brignon *et al.*, 1985) and porcine (Cerning-Beroard & Zevaco, 1984) κ-casein. In these two cases all or nearly all of the threonine residues in the macropeptide part of the molecules are glycosylated (Cerning-Beroard & Zevaco, 1984).

Phosphorylation of bovine κ-casein occurs on Ser-127, Ser-149 and in an unknown position. Although the average number of phosphate groups is approximately equal to the average number of GalNAc groups, i.e. about one group per κ-casein molecule, the distribution of phosphate groups is completely different from that of the carbohydrate over the κ-casein molecules. Ser-149 is always phosphorylated and therefore no phosphate-free κ-casein exists, which precludes a random phosphorylation mechanism. In this case the sites are apparently far from equivalent; the residence time in the Golgi apparatus is long enough to saturate the Ser-149, but only partial modification of the other sites occurs during this period.

According to Takeuchi *et al.* (1984) the following sequence takes place in the lactating cell of the Golgi apparatus: phosphorylation of casein, assembly of casein micelles, glycosylation of κ-casein on the surface of the micelle. Since we found only multiply phosphorylated fractions without carbohydrate, one could infer that phosphorylation of Ser-127 inhibits glycosylation *in vivo*.

Determination of kinetics

The h.p.g.p.c. method used for determining the kinetics of the cleavage of κ-casein led to reproducible results only when very strict precautions were taken. Especially, adsorption of protein to the column material formed a serious problem, which could be circumvented by a careful conditioning procedure. When comparing the kinetic parameters of the cleavage of κ-CN B-1 casein with those found previously (Visser *et al.*, 1980) under similar conditions for a tetradeca- or pentadeca-peptide containing the κ-casein sequence around its chymosin-sensitive 105–106 peptide linkage, one observes that the proteolytic coefficient, $k_{cat.}/K_m$, for κ-CN B-1 casein is only a factor of 2 or 3 larger; this is exclusively due to a lower K_m value. Also, as reported previously (Visser *et al.*, 1980), the $k_{cat.}/K_m$ values of about 2 $\mu M^{-1} \cdot s^{-1}$ found at pH 6.6 for the above peptide fragments are even closer to the one found for the cleavage of whole κ-casein (cf. Table 2).

It is noteworthy that we did not find any indication of substrate inhibition during the cleavage of κ-CN B-1 casein up to an initial substrate concentration of 400 μM. The latter is in contrast with what has been reported for the action of chymosin on κ-casein by Sanner & Kovács-Proszt (1973). Those authors incorrectly suggested a parallel with an inhibition model established for other enzyme–substrate systems. From the results in Table 2 it is seen that the kinetic parameters found for κ-CN B-1, κ-CN B-5 and κ-CN B-7 differ only to a relatively small extent. There exists a decrease in $V_{max.}$ when going to κ-casein components of higher NeuAc content.

The critical micelle concentration for κ-CN B-1 (25 μM) is in the middle of the range (3–45 μM) that was ordinarily used in our kinetic experiments. There are no indications from the v-against-[S] curves that there is a break in the properties at 25 μM. This could suggest that the accessibility of the Phe^{105}–Met^{106} linkage in the κ-casein molecule does not change when monomer κ-casein becomes part of the polymer and that the difference in size of the substrate plays a minor role.

When a solution of irreversible 12 S κ-casein polymers was used as a substrate, it was found that the parameters change only a little (cf. Table 2).

For 22 S particles, both $k_{cat.}$ and K_m become twice as large, but their ratio ($k_{cat.}/K_m$) is the same as for the 12 S particles, i.e. 3 $\mu M^{-1} \cdot s^{-1}$.

A solution of whole κ-casein that has not been brought into contact with 2-mercaptoethanol usually has particles with a broad size distribution with an average size distribution somewhere between the 12 S and 22 S particles. Both $k_{cat.}$ and K_m have increased somewhat compared with the 22 S particles, but the ratio $k_{cat.}/K_m$ does not change (Table 2). The $k_{cat.}/K_m$ values reported here are a factor of 10–100 lower than the limit above which collision between substrate and enzyme is generally taken to be diffusion-controlled (Fersht, 1984).

In all these cases the substrate particles were much smaller than casein micelles in milk, and it is interesting to compare our results with those found for bovine milk. Since bovine milk contains about 160 μM-κ-casein, the situation therein is likely to be governed by near-$V_{max.}$ conditions.

Van Hooydonk *et al.* (1984) determined the release of κ-casein macropeptide from casein micelles as a function of time in a milk-protein concentration range in which the κ-casein concentration was 160–400 μM. From the initial rate, which can be calculated from the progression curves given by Van Hooydonk *et al.* (1984), it is possible to construct v-against-[S] plots for the different milk batches which were used. It appears that v is approximately constant for [S] between 250 and 400 μM, but the extent of this constant region depends somewhat on the batch. From these v values, which are apparently $V_{max.}$ values, the $k_{cat.}$ is found to be between 70 and 90 s^{-1} for the different milk batches. This is close to the value we found for κ-CN B (cf. Table 2). A rough estimate of the upper limit of K_m from the v-against-[S] plots for milk is approx. 100 μM.

Castle & Wheelock (1972) found, for different batches of milk, widely differing K_m values, which was ascribed to the varied carbohydrate content of κ-casein in these milks. Van Hooydonk *et al.* (1984) reported a lower rate of macropeptide release with increasing carbohydrate content of the milk. On the other hand, Chaplin & Green (1980) and also Gibbons & Cheeseman (1962) reported that the carbohydrate content of κ-casein has no impact on the kinetics of its cleavage by chymosin in milk.

Data for kinetic parameters reported by others for the action of chymosin on isolated whole κ-casein are rather scarce (see also Table 2) and are always determined by procedures and/or under conditions which are different from ours. Some of these results are in reasonable agreement with the ones presented in here (cf. Table 2).

For isolated κ-casein fractions with varied carbohydrate contents, Doi *et al.* (1979*a*) and Addeo *et al.* (1984) found a decrease of rate of release of macropeptide with higher NeuAc content of the κ-casein fraction. Because of the rather high substrate concentrations Doi *et al.* (1979*a*) and Addeo *et al.* (1984) employed, their rate constants were probably close-to-$V_{max.}$ values; the decrease in rate of release with higher NeuAc content would then be in line with our results.

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REFERENCES

Addeo, F., Martin, P. & Ribadeau-Dumas, B. (1984) Milchwissenschaft **39**, 202–205
Azuma, N., Kaminogawa, S. & Yamauchi, K. (1984) Agric. Biol. Chem. **48**, 2025–2031
Brignon, G., Chtourou, A. & Ribadeau-Dumas, B. (1985) FEBS Lett. **188**, 48–54
Carles, C. & Ribadeau-Dumas, B. (1985) FEBS Lett. **185**, 282–286
Castle, A. V. & Wheelock, J. V. (1972) J. Dairy Res. **39**, 15–22
Cerning-Beroard, J. & Zevaco, C. (1984) J. Dairy Res. **51**, 259–266
Chaplin, B. & Green, M. L. (1980) J. Dairy Res. **47**, 351–358
Dalgleish, D. G. (1982) in Developments in Dairy Chemistry (Fox, P. F., ed.), part 1, pp. 157–187, Applied Science Publishers, London
De Koning, P. J. (1968) Neth. Milk Dairy J. **22**, 121–124
Doi, H., Kawaguchi, N., Ibuki, F. & Kanamori, M. (1979*a*) J. Nutr. Sci. Vitaminol. **25**, 33–41
Doi, H., Ibuki, F. & Kanamori, M. (1979*b*) J. Dairy Sci. **62**, 195–203
Dowd, J. E. & Riggs, D. S. (1965) J. Biol. Chem. **240**, 863–869
Eigel, W. H., Butler, J. E., Ernstrom, C. A., Farrell, H. M., Jr., Harwalkar, V. R., Jenness, R. & Whitney, R. McL. (1984) J. Dairy Sci. **67**, 1599–1631
Fersht, A. (1984) Enzyme Structure and Function 2nd edn., pp. 147–154, W. H. Freeman and Co., New York
Foltmann, B. (1971) in Milk Proteins (McKenzie, H. A., ed.), vol. 2, pp. 217–254, Academic Press, New York
Foltmann, B., Barkholt-Pedersen, V., Jacobsen, H., Kauffman, D. & Wybrandt, G. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 2321–2324
Friedenthal, M. K. & Visser, S. (1985) Neth. Milk Dairy J. **39**, 63–70
Garnier, J., Mocquot, G., Ribadeau-Dumas, B. & Maubois, J. L. (1968) Ann. Nutr. Aliment. **22**, B495–B552
Gibbons, R. A. & Cheeseman, G. C. (1962) Biochim. Biophys. Acta **56**, 354–356
Griswold, B. L., Humoller, F. L. & McIntyre, A. R. (1951) Anal. Chem. **23**, 192–194
Hanes, C. S. (1932) Biochem J. **26**, 1406–1421
Horne, D. S. (1984) J. Colloid. Interface Sci. **98**, 537–548
Lineweaver, H. & Burk, D. (1934) J. Am. Chem. Soc. **56**, 658–666
Mercier, J.-C. (1981) Biochimie **63**, 1–17

Mercier, J.-C., Maubois, J. L., Poznanski, S. & Ribadeau-Dumas, B. (1968) Bull. Soc. Chim. Biol. **50**, 521–530
McKenzie, H. A. & Wake, R. G. (1961) Biochim. Biophys. Acta **47**, 240–242
Sanner, T. & Kovács-Proszt, G. (1973) Biochim. Biophys. Acta **303**, 68–76
Schmidt, D. G. (1964) Biochim. Biophys. Acta **90**, 411–414
Sinkinson, G. & Wheelock, J. V. (1970) Biochim. Biophys. Acta **215**, 517–521
Slattery, C. W. (1978) Biochemistry **17**, 1100–1104
Takeuchi, M., Tsuda, E., Yoshikawa, M., Sasaki, R. & Chiba, H. (1984) Agric. Biol. Chem. **48**, 2789–2797
Van Halbeek, H., Dorland, L., Vliegenthart, J. F. G., Fiat, A.-M. & Jollès, P. (1980) Biochim. Biophys. Acta **623**, 295–300
Van Hooydonk, A. C. M. & Olieman, C. (1982) Neth. Milk Dairy J. **36**, 153–158
Van Hooydonk, A. C. M., Olieman, C. & Hagedoorn, H. G. (1984) Neth. Milk Dairy J. **38**, 207–222
Visser, S. & Rollema, H. S. (1986) Anal. Biochem. **153**, 235–241
Visser, S., Van Rooijen, P. J., Schattenkerk, C. & Kerling, K. E. T. (1976) Biochim. Biophys. Acta **438**, 265–272
Visser, S., Van Rooijen, P. J., Schattenkerk, C. & Kerling, K. E. T. (1977) Biochim. Biophys. Acta **481**, 171–176
Visser, S., Van Rooijen, P. J. & Slangen, Ch. J. (1980) Eur. J. Biochem. **108**, 415–421
Vreeman, H. J., Both, P., Brinkhuis, J. A. & Van der Spek, C. A. (1977) Biochim. Biophys. Acta **491**, 93–103
Vreeman, H. J., Brinkhuis, J. A. & Van der Spek, C. A. (1981) Biophys. Chem. **14**, 185–193
Warren, L. (1959) J. Biol. Chem. **234**, 1971–1975
Wheelock, J. V., Sinkinson, G. & Hindle, E. J. (1972) Dairy Sci. Abstr. **34**, 655–664
Yamauchi, K., Azuma, N., Kobayashi, H. & Kaminogawa, S. (1981) J. Biochem. **90**, 1005–1012
Zevaco, C. & Ribadeau-Dumas, B. (1984) Milchwissenschaft **39**, 206–210

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