Carbohydrate Polymers 7 (1987) 361–369

Studies of Camel Casein Micelles: Treatment with Soluble and Immobilized Neuraminidase

Mohamed A. Mehaia

Food Biotechnology Laboratory, Department of Food Science, College of Agriculture, PO Box 2460, King Saud University, Riyadh 11451, Saudi Arabia

(Received 6 January 1987; accepted 2 February 1987)

SUMMARY

Camel casein micelles were obtained from raw uncooled skim milk by ultracentrifugation, washing and then resuspending in UF-skim milk permeate. They were treated with neuraminidase, in both soluble and immobilized forms, to study the location and distribution of the glycosylated portion of camel casein micelle. Kinetic release of sialic acid, soluble in 12% TCA, was studied. Camel casein micelle contains 7·35 mg sialic acid per g casein; 99·3% of it was released with soluble neuraminidase, whereas only 90% was released with immobilized neuraminidase. This implies that about 90% of the glycosylated portion of camel casein micelle (glyco-κ-casein-like component) is on the surface of the micelle.

INTRODUCTION

Casein is synthesized in the secretory cells of the mammary gland in a highly aggregated state making more or less spherical micelles ranging, in camel milk, from 14 to 560 nm in diameter (Ali & Robinson, 1985). The structure and properties of these micelles largely determine the behaviour of milk during technological processes such as pasteurization, sterilization and curdling for cheese manufacture. Caseins are the most abundant proteins (> 60% in camel) (Mehaia, unpublished data) in most mammalian milks and consist primarily of a group of heterogeneous glyco-phospho-protein (Eigel *et al*, 1984).

The size, stability and structure of bovine casein micelles have been extensively investigated and several models have been proposed (Slattery, 1976; Schmidt, 1980; Payens, 1982; Mehaia, 1983; McMahon & Brown, 1984; Holt, 1985). The casein micelles isolated from milk of

Carbohydrate Polymers 0144-8617/87/$03.50 —

some other mammals have also been studied (Jolles, 1975; Addeo *et al.*, 1978; Jenness, 1982; Cerning-Beroard & Zevaco, 1985; Ono & Creamer, 1986). In bovine casein micelles it is considered that the four major casein components (α_{s1}-, α_{s2}-, β- and *k*-casein) form aggregates or submicelles, which coalesce together with mineral material to form the casein micelles (Schmidt, 1980; Payens, 1982; Mehaia, 1983; McMahon & Brown, 1984). The *k*-casein fraction is the key to the stability of the casein micelle; it is also the only casein fraction which contains sugar. There are two forms of *k*-casein, one containing varying amounts of carbohydrate moiety, i.e. sialic acid, known as 'glyco-*k*-casein', and another devoid of carbohydrates, known as 'nonglyco-*k*-casein' (Mehaia, 1983; Mehaia & Cheryan, 1983*a*; Eigel *et al.*, 1984; McMahon & Brown, 1984). Several workers consider the sialic acid as contributing significantly to the 'stability' of the *k*-casein and thus to the stability of the micelle (Beedy & Nitschmann, 1963; Wheelock & Knight, 1969; Sinkinson & Wheelock, 1970).

Little, however, is known about the major components and the structure of camel casein micelle (Dedek *et al.*, 1978; Farah & Farah-Riesen, 1985; Mehaia, 1987*a*, 1987*b*). Although *k*-casein has never been isolated in a pure state from camel milk, the addition of chymosin to camel milk caused a clotting reaction with a coagulum (Farah & Farah-Riesen, 1985; Mehaia, 1987*a*, 1987*b*). This suggests the possible occurrence of *k*-casein or a homologous protein in camel milk. As part of the continuing studies on camel casein micelles (Mehaia, 1987*a*, 1987*b*), a study is reported here of the location and distribution of the glycosylated portion of camel casein micelle (glyco-*k*-casein-like component) using soluble and immobilized neuraminidase. Soluble neuraminidase is expected to penetrate the micelle and react with all the glycosylated portion of the micelle (Fig. 1A), whereas immobilized neuraminidase, being much larger than the casein micelle, will not penetrate it but instead react only with the micelle surface (Fig. 1B).

MATERIALS AND METHODS

Enzymes

Neuraminidase (Sialidase, EC 3.2.1.18, Catalog no. N-2876) and immobilized neuraminidase (insoluble neuraminidase attached to beaded agarose, Catalog no. N-5254) were purchased from the Sigma Chemical Company, St Louis, MO, USA.

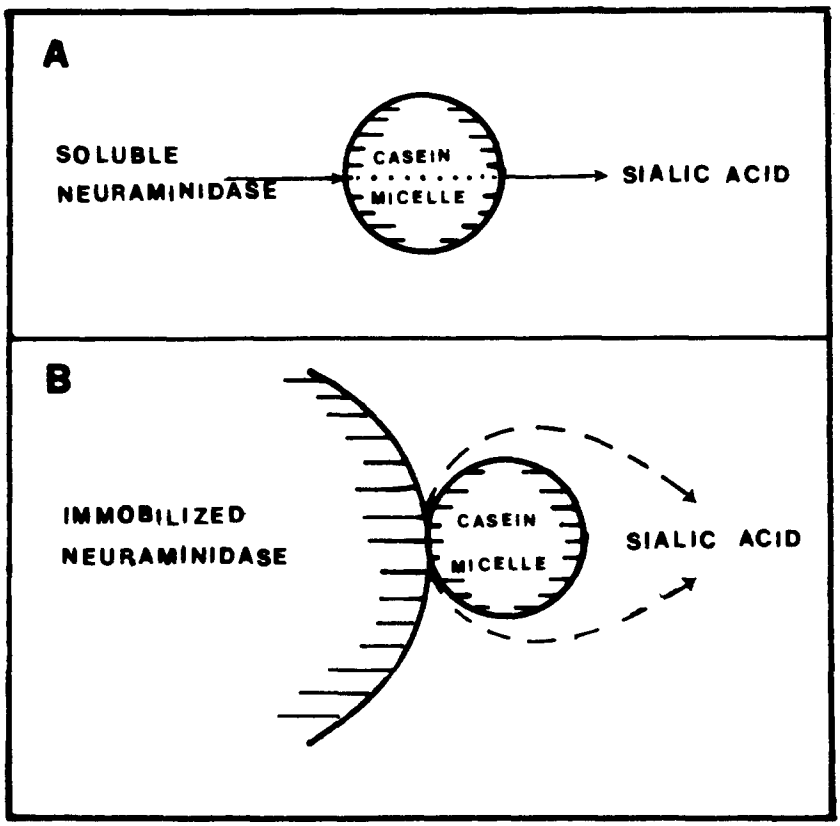

Fig. 1. Schematic representation of sialic acid released from camel casein micelle by the action of (A) soluble neuraminidase, (B) immobilized neuraminidase.

Preparation of casein micelles

Fresh milk was obtained from one to five Najdi camels (*Camelus dromedarius*) from the private farm near Riyadh City, Saudi Arabia. The milk was centrifuged at 4000 g for 30 min to separate fat and sludge. The skim milk was then centrifuged at 60 000 g for 60 min in a Beckman Model L8-80 ultracentrifuge. The supernatant fluid was discarded, and the skim milk pellet was twice washed by suspending it in the UF-permeate of the same skim milk, with a Soniprep 150-MSE Tissue Homogenizer, and recentrifuging. After the last wash the pellet was resuspended in UF-permeate to a normal casein level of about 2 g 100 ml^{-1}. Ultrafiltration of skim milk was carried out at 30°C by using a hollow fibre from Amicon (Danvers, MA, USA) and H1P1-43 membrane. To prevent dissociation of the micelle, care was taken throughout

the experiments to avoid cooling the milk or micelles below 20°C. To minimize microbial growth, thimerosal (Sigma Chemical Co.) was used at a concentration of 100 ppm in the reaction mixture. It was effective in controlling microbial problems while having no detectable effect on enzyme activity at this concentration (Mehaia & Cheryan, 1983*a*).

Enzyme treatment of micelles

One hundred millilitres of the resuspended isolated casein micelles were placed in a 500 ml Erlenmeyer flask and equilibrated to 37°C, pH 6·6, in an agitated water bath. The enzyme, either 50 mg soluble neuraminidase or 4 ml immobilized neuraminidase, was added and the mixture stirred gently with a stirring rod for a few seconds. The reciprocating motion of the agitated water bath was adjusted to provide adequate agitation without excessive foaming. At various times, 10 ml of the reaction mixture was removed and 40 ml of 15% trichloroacetic acid (TCA) added to give 12% concentration. Samples were filtered through Whatman no. 42 filter paper, and the filtrate was analysed for sialic acid.

Control experiments

Controls were prepared to test for any solubilization of the immobilized neuraminidase, for any microbial activity and for any solubilization of micelles over the reaction period, as described by Mehaia and Cheryan (1983*a*).

Analytical methods

Protein and nitrogen (N) were determined using the Micro-Kjedahl test. Unless otherwise mentioned, the protein content is N × 6·38. Sialic acid was measured using resorcinol (Svennerholm, 1957) and thiobarbituric acid (Warren, 1959) methods. A standard curve was prepared with *n*-acetylneuraminic acid (Catalog no. A-2388, Sigma Chemical Co.).

RESULTS AND DISCUSSION

Sialic acid released by the action of soluble and immobilized neuraminidase

N-acetylneuraminic acid (NANA), more commonly known as a sialic acid, is an oligosaccharide component of the caseinoglycoprotein

(*k*-casein), and it is considered to be the major carbohydrate in *k*-casein (Jolles, 1975; Fournet *et al.*, 1979). The action of neuraminidase on the sialic acid (NANA) in *k*-casein is evidence that it occupies a terminal position in the *k*-caseinoglycopeptide part (Alais & Jolles, 1961; Jolles *et al.*, 1969; Fournet *et al.*, 1979).

Figure 2 shows the release of sialic acid by the action of soluble neuraminidase at 37°C and pH 6·6, each point is the mean of three replicate experiments. The total sialic acid concentration in the casein micelles was 7·35 mg g^{-1} casein. In Fig. 2 an initial rapid release of sialic acid is followed by a levelling off, after about 18 h, at a maximum value corresponding to 99·3% of the total sialic acid of casein micelles.

The action of immobilized neuraminidase on casein micelles is shown in Fig. 3. The initial rate of sialic acid release with immobilized neuraminidase was slower than that of soluble neuraminidase. Maximum sialic acid released (total sialic acid minus sialic acid at zero time and sialic acid from control experiments), after 42 h, was 6·6 mg g^{-1} casein. This is about 90% of the total sialic acid of casein micelles. This means

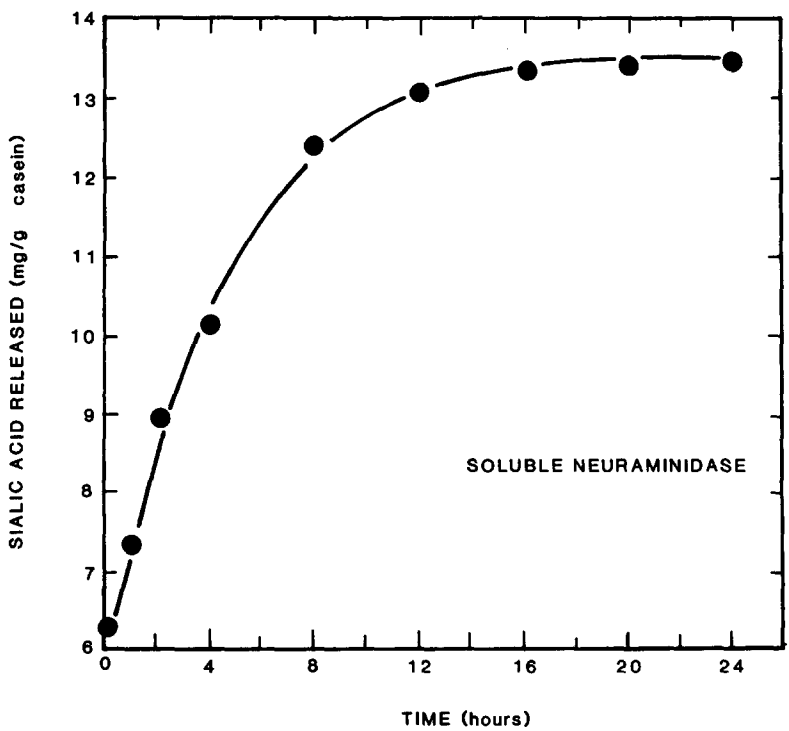

Fig. 2. Release of sialic acid soluble in 12% TCA from camel casein micelles by soluble neuraminidase at pH 6·6, 37°C.

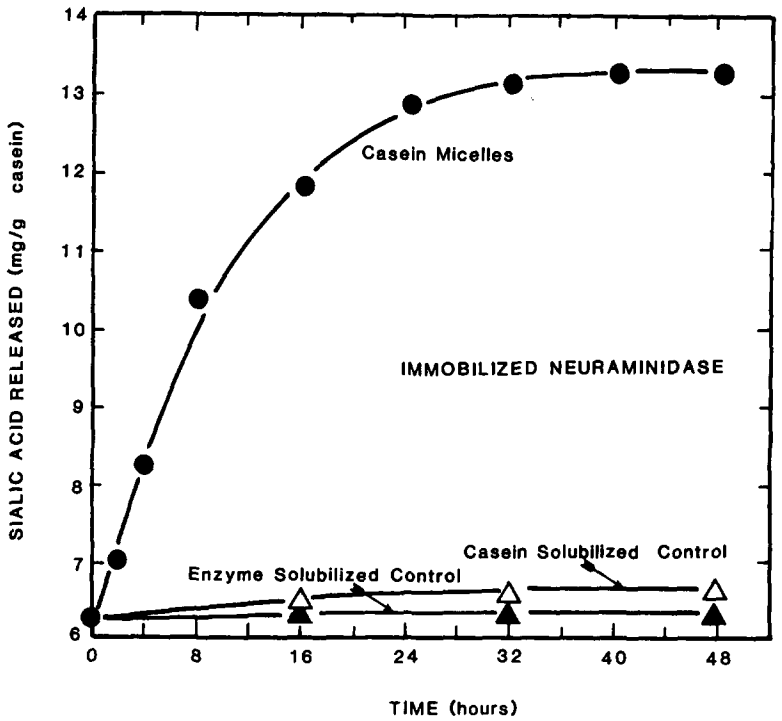

Fig. 3. Release of sialic acid soluble in 12% TCA by the action of immobilized neuraminidase at pH 6·6, 37°C.

that the maximum sialic acid released by immobilized neuraminidase was about 90% of that released by soluble neuraminidase. Since immobilized neuraminidase cannot penetrate the casein micelles, it suggests that most (90%) of the glycosylated portion of camel casein micelle is on the surface of the micelle.

Control experiments

Tests for microbial activity indicated that there was no detectable increase in nonprotein nitrogen in the TCA filtrates, indicating that there was no significant proteolytic and/or microbial activity, and all the sialic acid was from neuraminidase activity.

Test for 'soluble' activity of immobilized neuraminidase (enzyme solubilized control, Fig. 3) revealed that there was no increase of sialic acid during the reaction time, indicating that there was essentially no free neuraminidase activity in the immobilized enzyme.

Test for solubilization of casein micelles (casein solubilized control, Fig. 3) indicated that there was a slight increase in sialic acid (about 4% of the total amount) during the time of the experiment (48 h). Thus, a net release of the sialic acid from the casein micelle itself was obtained by subtracting the sialic acid of the casein solubilized control from the total sialic acid released.

SUMMARY AND CONCLUSION

The advent of immobilized enzyme technology has brought about the development of a specific reagent that would not penetrate the micelle, but would instead interact only with the surface. Recently, several studies have shown the value of this technique in elucidating the surface structure of the casein micelle (Ashoor *et al.*, 1971; Cheryan *et al.*, 1975) and the location and distribution of glycosylated *k*-casein. (Mehaia & Cheryan, 1983*a*, 1983*b*; Mehaia, 1987*a*, 1987*b*). Table 1 shows a comparison of sialic acid released from bovine and camel casein micelle by the action of soluble and immobilized enzymes. Isolated camel casein micelle contains more sialic acid (glyco-*k*-casein-like component) than does bovine casein micelle (glyco-*k*-casein). Comparison of Figs 1 and 2 indicates that immobilized neuraminidase released about 90% of the sialic acid which the soluble neuraminidase released (Table 1). This

TABLE 1
Comparison of Sialic Acid Released from Bovine and Camel Casein Micelle by the Action of Soluble and Immobilized Enzymes

Casein micelle	*Enzyme*	*Sialic acid released (mg g^{-1} casein)*			
		Soluble enzyme (SE)	*Immobilized enzyme (IE)*	*IE/SE*	*Reference*
Bovine[a]	Chymosin	2·70	2·31	0·85	Mehaia & Cheryan, 1983*b*
	Pepsin	2·74	2·42	0·88	Mehaia & Cheryan, 1983*b*
	Neuraminidase	3·00	2·30	0·76	Mehaia & Cheryan, 1983*a*
Camel[b]	Chymosin	7·20	6·80	0·94	Mehaia, 1987*a*
	Pepsin	7·10	6·70	0·94	Mehaia, 1987*b*
	Neuraminidase	7·30	6·60	0·90	This work

[a]Sialic acid content of bovine casein micelle = 3·02 mg g^{-1} casein.
[b]Sialic acid content of camel casein micelle = 7·35 mg g^{-1} casein.

implies that about 90% of the glycosylated portion of camel casein micelle is on the surface of the micelle, which corroborates the protease-micelle studies (Mehaia, 1987*a*, 1987*b*). This is similar to the results obtained from the previous studies on bovine casein micelle (Table 1). However, camel casein micelles showed no sign of forming a clot or precipitate during neuraminidase action, even after 99% of the total NANA was released. Thus it appears that carbohydrate moiety *per se* may not be the key factor controlling the stability of the micelle; this agrees with several other studies (Gibbons & Cheeseman, 1962; Mackinley & Wake, 1965; Mehaia & Cheryan, 1983*a*). However more studies are needed to clarify the structure of camel casein micelle to explain the nature of camel milk casein and its chemical and physical behaviour.

ACKNOWLEDGEMENTS

This research was supported by the Agriculture Research Center, College of Agriculture, King Saud University, Saudi Arabia. Skilful technical assistance by Mr A. M. Abou El-Kheir and Mr M. A. Hablas is highly appreciated.

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