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MILK FAT GLOBULE MEMBRANES
INHIBITION BY SUCROSE OF THE ALKALINE PHOSPHOMONOESTERASE

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SUMMARY

Sucrose, a widely used agent in the preparation of membranes, inhibited the alkaline phosphomonoesterase of the milk fat globule membrane in both its membrane-bound and detergent-solubilized forms. The inhibition was kinetically competitive and reversible by dialysis. However, its mechanism was more complex than simple competition with substrate because: (a) sucrose induced the appearance of prolonged time-lags in the progress curves of the enzyme; (b) the extent of inhibition and of the time-lags depended on the age of the membrane preparation, the period of pre-exposure of the membranes to sucrose, and the temperature of pre-exposure. On the other hand the acid phosphomonoesterase and the phosphodiesterase activities also present in the membrane preparations were unaffected by the disaccharide.

INTRODUCTION

Milk fat globule membranes are partly derived from the plasma membrane of the acinar cells of the mammary gland. They can be obtained in large amounts from fresh milk and are a convenient source of membrane-bound enzymes. We are interested in the comparative study of the membrane-bound and soluble forms of phosphoesterases, but are faced with the problem that the activity of these enzymes in milk fat globule membranes is subjected to wide variations, especially the alkaline phosphomonoesterase [1–3]. These variations could be the product of many factors including a period of lactation of the animals and conditions of preparations of the membranes. One such factor is sucrose, that is frequently included in the preparation and storage of the milk fat globule membranes [1, 4–6]. In an early study of the phosphotransferase activity of soluble alkaline phosphomonoesterase from cow's milk and intestinal mucose Morton [7] found, using creatine-phosphate as phosphoryl donor, that sucrose was not an acceptor in the phosphate transfer reaction but was a weak inhibitor of the hydrolysis of the donor. We show in this paper that sucrose exerts a strong inhibitory action on the membrane-bound alkaline phosphomonoesterase and,

furthermore, that its inhibitory effect depends upon age, storage conditions and time of exposure of the membrane to the disaccharide. The effect of sucrose on the activity and stability of the acid phosphomonoesterase and the phosphodiesterase was also studied for comparative purposes.

MATERIALS AND METHODS

Chemicals

The sucrose used was obtained from Merck (Darmstadt, G.F.R., analytical grade) and Carlo Erba (Milano, Italy, analytical grade). Three different batches of each were tried with similar results. *p*-Nitrophenylphosphate (disodium salt), bis-*p*-nitrophenylphosphate (hemi calcium salt), and Triton X-100 were purchased from the Sigma Chem. Co. (St. Louis, Mo.). All other reagents were of the best quality commercially available.

Preparation of membrane material

Friesian cows free from mastitis were from the Experimental Farm of this Institute. Samples of milk from individual cows were collected from the morning milking (5–6.00 a.m.) and transferred to the laboratory to be processed immediately (about 10 min after milking). The cream from 5–10 l of milk was separated at 28–32 °C using a Westfalia DB cream separator, washed twice at 5 °C by suspension in three volumes of 10 mM imidazol-HCl buffer (pH 7.1) containing 0.25 M sucrose and 2 mM $MgCl_2$ and centrifugation at $4000 \times g$ for 10 min. The washed cream was adjusted to 25–30 % fat by suspension in the previous buffer containing in addition 2 mM 2-mercaptoethanol (cream/buffer, 1 : 2, w/v) and churned. After straining through cheese cloth, the buttermilk (about 0.5 mg protein/ml) was centrifuged at $40\,000 \times g$ for 30 min at 2 °C to give a pellet milk fat globule membranes and a supernatant. The brown pellet (milk fat globule membranes) was collected, washed twice with 10 mM imidazol HCl (pH 7.1) containing 0.25 mM sucrose, 2 mM $MgCl_2$ and 2 mM 2-mercaptoethanol and finally suspended in the same buffer to a final protein concentration of 10–20 mg/ml. The above buffer without sucrose will be referred to as standard buffer. Milk fat globule membranes were also obtained without using sucrose at any stage of the isolation, other steps being identical to those described above. When direct comparison was desired, the cream was divided into two equal volumes and processed as described with and without sucrose. The fat globule had the same stability and could be broken equally well in the presence or absence of sucrose, but the isolated membranes aggregated strongly when stored at −20 °C in the absence of disaccharide.

Enzymic assays

Acid phosphomonoesterase (EC 3.1.3.2) and phosphodiesterase (EC 3.1.4.1) were assayed as described by Ostrowski and Tsugita [10]. Alkaline phosphomonoesterase (EC 3.1.3.1) was assayed as the acid phosphomonoesterase but using as incubation buffer 100 mM glycine-NaOH (pH 10.5). The nitrophenol released was estimated spectrophotometrically using an extinction coefficient of 18 000 at 400 nm. Activity was expressed as nmol of *p*-nitrophenol released per mg of protein per min.

TABLE I

EFFECT OF THE STORAGE AT −20 °C ON THE MILK FAT GLOBULE MEMBRANES PHOSPHOESTERASE ACTIVITIES AND THE INFLUENCE OF SUCROSE

The membranes were prepared, washed and stored in the presence or absence of 0.25 M sucrose as indicated in the text. The enzymes were assayed as described in Methods. Final substrate concentrations were 1 mM (alkaline phosphomonoesterase), 8 mM (acid phosphomonoesterase) and 2.5 mM (phosphodiesterase). The membrane suspensions were thawed only once, i.e., when necessary to assay their enzyme activities.

Conditions of preparation	Time of storage at 20 °C (days)	Alkaline phosphomonoesterase		Acid phosphomonoesterase		Phosphodiesterase	
		Spec. act. (nmol · mg · min)	% initial activity	Spec. act. (nmol · mg · min)	% initial activity	Spec. act. (nmol · mg · min)	% initial activity
No sucrose							
	0	171	100	6.2	100	68	100
	9	143	84	6.0	97	67	98.5
	21	137	80	5.9	95	56	82
	30	130	76	5.8	94	48	70
	120	–	–	2.4	38	–	–
0.25 sucrose							
	0	80	100	5.4	100	52	100
	9	74	93	5.0	93	51	98
	21	76	95	5.3	98	48	92
	30	64	80	5.2	96	51	98
	120	–	–	1.8	35	51	98

RESULTS AND DISCUSSION

Stability of the phosphoesterase activities during storage at −20 °C and to repeated thawing. Influence of sucrose

Milk fat globule membranes were obtained in the presence and the absence of sucrose, and their alkaline and acid phosphomonoesterase and phosphodiesterase activities determined after storage at −20 °C for various periods of time. As shown in Table I the three phosphoesterase activities were fairly stable. After 1 month at −20 °C, 70–95 % of the activities remained both in the absence and the presence of sucrose. The enzyme most sensitive to storage under these conditions was the acid phosphomonoesterase: it retained only 35–38 % of its activity after 4 months at −20 °C. Sucrose did not influence the stability of the alkaline phosphomonoesterase activity, but in the membranes prepared and stored in the presence of the sugar the enzyme had a lower specific activity.

Repeated freeze-thawing cycles had a more profound effect on the alkaline phosphomonoesterase than storage. Regardless of the period of storage at −20 °C, a membrane suspension thawed 3–4 times, lost about 40–50 % of its activity (Table II). Sucrose had only a slight protective effect. On the other hand, the acid phosphomonoesterase and phosphodiesterase activities were virtually unaffected by thawing the suspension up to 4-times in the presence or the absence of sucrose.

Effect of washing with buffer containing or not sucrose on the phosphoesterase activities of the milk fat globule membranes

The two washings included in the method of preparation of the milk fat globule membranes resulted in the removal of about 25 % of the initial milk fat

TABLE II

EFFECT OF THAWING ON THE ALKALINE PHOSPHOMONOESTERASE ACTIVITY OF THE MILK FAT GLOBULE MEMBRANES STORED AT −20 °C

Freshly prepared membranes, washed and suspended in 10 mM imidazol-HCl pH 7.0 containing 2 mM $MgCl_2$, 2 mM 2-mercaptoethanol and 0.25 M or no sucrose, were assayed for alkaline phosphomonoesterase activity using a final concentration of substrate 1 mM. Membranes were then stored frozen at −20 °C, thawed to determine enzymic activity and frozen again after about 4 h. The experiment reported was carried out using milk fat globule membranes obtained from a cow in its fifth month of lactation. Similar results were obtained from milk of another three cows in periods of lactation ranging from the third to the eight month. n.d., not determined.

No of times thawed	Time of storage at −20 °C (days)	No sucrose		0.25 M sucrose	
		Spec. act.	% of initial act.	Spec. act.	% of initial act.
0	*	190	100	29	100
1	6	159	84	27	93
2	12	133	70	22	76
3	20	116	61	19	66
4	26	90	47	16.5	57
4	120	80	42	n.d.	n.d.

* Fresh enzyme.

TABLE III

INFLUENCE OF WASHINGS ON THE MEMBRANE-BOUND PHOSPHOESTERASE ACTIVITIES

The membranes were washed by suspension (60 ml, 0.5 mg protein/ml) in 10 mM imidazol-HCl buffer (pH 7.1) containing 2 mM Mg^{2+}, 2 mM 2-mercaptoethanol with or without 0.25 M sucrose, and centrifuged at 40 000 × *g* for 30 min at 2 °C. The pellet was suspended to the same volume with buffer, gently homogenized (potter 5–8 strokes) and the centrifugation repeated. All operations were carried out in an ice-bath. The enzymes were assayed as described in Methods. Final substrate concentrations were 1.0 mM (alkaline phosphomonoesterase), 8.0 mM (acid phosphomonoesterase) and 2.5 mM (phosphodiesterase). The values reported for the specific activities correspond to a single cow in its third month of lactation and were variable from one animal to another. However, the percentage of total protein and percentage total enzymic activities were reproducible and the values reported (±S.D.) are the average for three different cows (figures in brackets).

No of washings	% protein that remains in the membrane	Enzyme activity remaining membrane-bound					
		Alkaline phosphomonoesterase		Acid phosphomonoesterase		Phosphodiesterase	
		Spec. act. (nmol/mg per min)	% of total activity	Spec. act. (nmol/mg per min)	% of total activity	Spec. act. (nmol/mg per min)	% of total activity
No sucrose							
0 (3)	100	64.0	100	4.7	100	41.3	100
1 (3)	90.5 ± 2	64.6	88 ± 2	5.0	86 ± 2	40.3	82.5 ± 4
2 (3)	75 ± 5	65.0	82 ± 7	5.0	80 ± 4	40.6	74 ± 6
0.25 M sucrose							
0 (3)	100	22.5	100	5.0	100	36.5	100
1 (3)	82.5 ± 2	14	51 ± 4	4.9	81 ± 2	32.5	75 ± 3
2 (3)	73.0 ± 1	12	26 ± 5	5.3	76 ± 2	33.5	64 ± 2

globule membranes protein irrespective of the presence of sucrose (Table III). In the absence of sucrose the enzymes followed the total protein distribution as reflected in their unchanged specific activities. However, in the presence of sucrose about 30 % of the phosphodiesterase and 60 % of the alkaline phosphomonoesterase appeared to be selectively removed. The phosphodiesterase activity lost from the milk fat globule membranes appeared in the supernatants, but the recovery of alkaline phosphomonoesterase was only 30–40 % of the total. Because unspecific inactivation of the enzyme seemed unlikely we examined the effect of adding sucrose to membranes prepared in its absence, as described below.

Reversible inhibition of alkaline phosphomonoesterase by sucrose

Milk fat globule membranes prepared in the absence of sucrose were suspended in standard buffer containing 0.25 M sucrose and alkaline phosphomonoesterase activity determined immediately or after maintaining the milk fat globule membranes for 24 h at −20 °C. Sucrose inhibited the enzyme by 20 % and storage at −20 °C in the presence of the sugar increased the inhibition to 80 %. Subjected to a similar treatment, the acid phosphomonoesterase and diesterase activities were unchanged with respect to controls to which sucrose had not been added.

To test the reversibility of the inhibition milk fat globule membranes (1.27 mg protein in 100 µl) obtained in the presence of 0.25 M sucrose were dialyzed against standard buffer (2 changes of 1 l) and alkaline phosphomonoesterase activity before and after dialysis was compared to that of milk fat globule membranes obtained without using sucrose at any time but treated in a similar manner. The inhibition was completely reversible. Although 50 % of the activity was lost by the control upon dialysis, the sucrose treated milk fat globule membranes gained three times its initial activity so that both control and experiment had nearly identical activities (30.3 and 30.6 nmol · mg^{-1} · min^{-1}, respectively).

Linearity with time in the presence and the absence of sucrose

In the absence of the inhibitor the enzymic activity was linear with time and protein concentration until about 50–60 % of the substrate had been hydrolyzed. However, in the membranes prepared in the presence of sucrose the enzyme activity showed a lag-period of 5–20 min. Both in the absence and in the presence of sucrose the substrate could be hydrolyzed almost totally (Fig. 1).

Factors influencing the inhibition by sucrose

The duration of the time-lag and the extent of inhibition caused by sucrose depended on the concentration of the sugar, the period and temperature of pre-incubation with it, and the age of the milk fat globule membranes preparation. Each of these factors is examined below in more detail.

Milk fat globule membranes pre-incubated with 4.5 mM sucrose for 30 min at 20 °C showed 33 % inhibition of its alkaline phosphomonoesterase activity (substrate 1 mM). However, no lag was seen until the sugar concentration reached 45 mM or higher (78 % inhibition). Increasing the sucrose concentration (0.45 M) did not produce a higher inhibition of the linear rate, but prolonged the duration of the lag.

The dependence of the inhibition on the period of pre-incubation with a fixed concentration of sucrose and the influence of the temperature are illustrated in Fig. 2

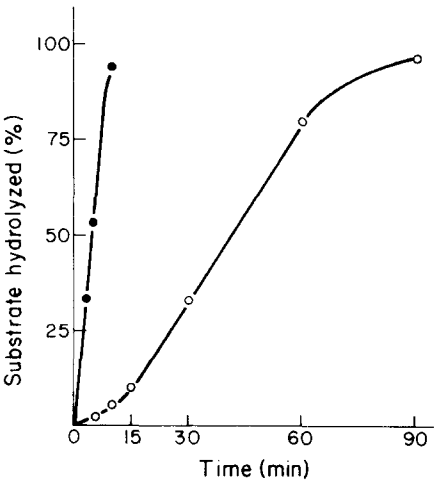

Fig. 1. Time-course of the hydrolysis of *p*-nitrophenylphosphate by alkaline phosphomonoesterase of milk fat globule membranes prepared (-●-), excluding sucrose; (-○-), in the presence of 0.25 M sucrose. The incubation mixtures contained in a final volume of 200 μl of 0.1 M glycine-NaOH buffer (pH 10.5), 1 mM substrate and 112 μg of milk fat globule membranes protein. Incubation was at 26 °C and the membranes were freshly prepared as described in Materials and Methods.

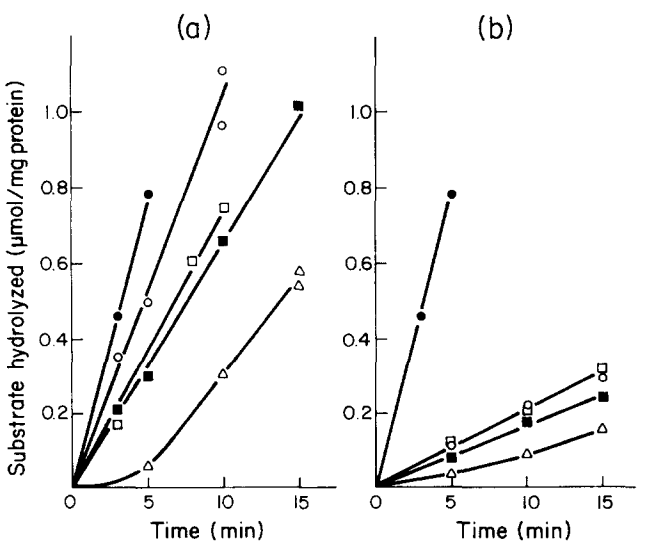

Fig. 2. Influence of the temperature and time of pre-incubation with sucrose on the time-course of the alkaline phosphomonoesterase. (a) Milk fat globule membranes (91 μg protein) immediately after preparation were pre-incubated at 0 °C with a final sucrose concentration of 38 mM for periods of: (-●-), no preincubation; (-○-), 15 min; (-□-), 30 min; (-■-), 60 min; (-△-), 120 min. (b) As for (a), but pre-incubation performed at 20 °C. Enzyme assay conditions as in Fig. 1.

(a and b). A period of pre-incubation of 2 h was necessary to show the development of a lag and maximum inhibition. Also, pre-incubation at 20 °C was much more effective in that respect than a similar treatment at 0 °C.

The inhibitory effect of sucrose was also dependent on the age of the milk fat globule membranes. After storage at −20 °C for 1 day the enzyme activity showed time lags after only 30 min pre-incubation with the sugar. However, if the storage was prolonged, the membrane-bound alkaline phosphomonoesterase became less sensitive to sucrose and, eventually, after 1 month storage at −20 °C, the inhibition reached a constant minimum value (57 %) and the lag was no longer observed.

Kinetics of alkaline phosphomonoesterase and K_i for sucrose

In membranes obtained in the absence of sucrose, the enzyme showed typical substrate inhibition behaviour (Fig. 3) in agreement with the results reported for the soluble form of the enzyme from a similar source [8, 9] or from other sources [11]. The value of K_m from the double-reciprocal plot (Fig. 3) at low substrate concentration was 0.6 mM. From a plot of $1/v$ against substrate concentration the value of K', the apparent dissociation constant for the second molecule of substrate [12] was estimated as 6.5 mM.

The K_i for sucrose was determined at low substrate concentrations, where the enzyme obeyed Michaelian kinetics. Fresh membranes, preincubated for 30 min at 20 °C with 0.45 to 45 mM inhibitor were used. Substrate concentration varied from 0.25 to 1 mM. Double-reciprocal or Dixon plots indicated that inhibition was competitive with a K_i value of 1.35 mM (Fig. 4).

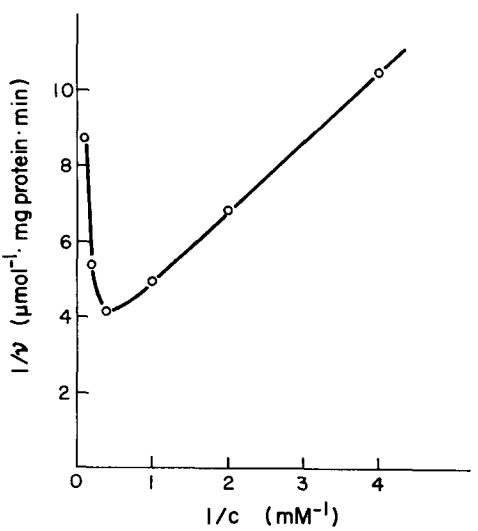

Fig. 3. Double-reciprocal plot for the alkaline phosphomonoesterase activity of the milk fat globule membranes. Assay conditions were as in Fig. 1. The reaction mixtures contained *p*-nitrophenylphosphate (0.25 to 10 mM) and membrane protein (110 μg) from milk fat globule membranes prepared 1–2 h before assay.

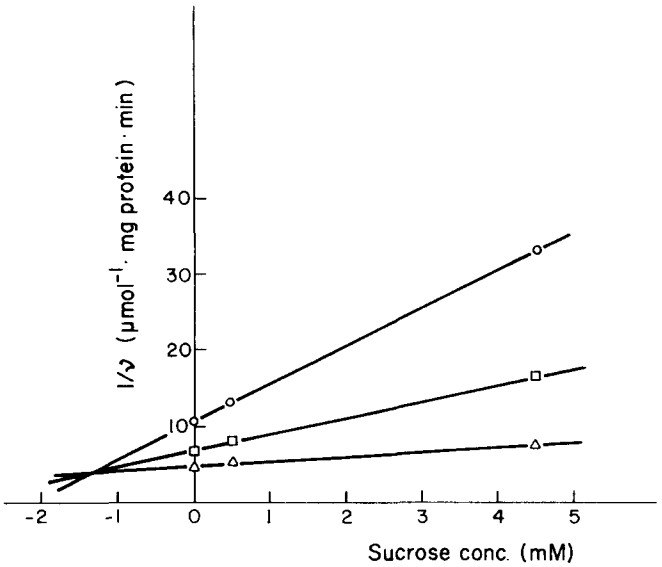

Fig. 4. Dixon's plot of the inhibition by sucrose of the milk fat globule membranes-bound alkaline phosphomonoesterase. The membranes (112 μg protein) were pre-incubated with the desired concentration of inhibitor for 30 min at 20 °C and then assayed as in Fig. 1 using as final substrate concentration: (-○-), 0.25 mM; (-□-), 0.5 mM; (-△-), 1 mM.

Inhibition by sucrose of alkaline phosphomonoesterase solubilized by Triton X-100

It was possible that the membrane structure or its alteration during storage could be responsible for some of the inhibitory effects of sucrose. Therefore, we studied the inhibition by the sugar after treating the milk fat globule membranes with

TABLE IV

ALKALINE PHOSPHOMONOESTERASE ACTIVITY. INHIBITION BY SUCROSE OF THE SOLUBILIZED AND PARTICULATE ENZYME

Membranes (15 mg protein/ml) were incubated in 10 mM imidazol-HCl (pH 7.0) containing 2 mM Mg^{2+}, 2 mM 2-mercaptoethanol and Triton X-100 (final concentration, 1 % w/v) for 1 h at pH 7 and 20 °C. The activity and inhibition by sucrose were then determined in the whole membrane-Triton suspension and in the supernatant and pellet of the centrifugation of the Triton-membrane mixture at 100 000 × *g* for 1 h. The same untreated membrane preparation was used as a control. The inhibition by sucrose was determined by pre-incubating the membrane preparations (50–110 μg of protein) with sucrose (final concentration 0.5 M) at 20 °C for 30 min in a final volume of 10 μl of 10 mM imidazol-HCl buffer (pH 7.0).

Preparation	Spec. act. (nmol/mg per min)	Inhibition by sucrose (%)
Control	110	79
Membrane-Triton	130	58
Supernatant	112	53
Pellet	145	68

Triton X-100 (detergent/protein, 0.67, w/w) for 1 h at 0 °C in standard buffer.

Under these conditions the detergent solubilized about 25 % of the membrane protein and the same amount of alkaline and acid phosphomonoesterase. The phosphodiesterase was more specifically removed (57 %). The specific activity of all the phosphoesterase activities increased by 20–30 % with respect to an untreated control. The complete inactivation of alkaline phosphomonoesterase observed by Mather and Keenan [13] was probably due to the high ratio of detergent to protein (10 : 1, w/w) used by these authors.

The effect of adding sucrose to whole milk fat globule membranes treated with Triton X-100, and to the supernatant and pellet resulting from centrifuging at $100\,000 \times g$ the detergent-treated membranes is compared in Table IV with an untreated control. The inhibition of alkaline phosphomonoesterase was little affected by the presence of detergent and the disorganization of the membrane structure.

CONCLUSION

Sucrose is widely used in the preparation of milk fat globule membranes because it prevents the strong aggregation of the membranes occurring upon storage or thawing and makes sampling of the suspension more reliable. However, it also causes a strong and complex inhibition of the alkaline phosphomonoesterase activity. This is particularly relevant to comparative studies of enzymic activities of milk fat globule membranes such as those of Dowben et al. [1] where this enzyme was one of those chosen. The inhibition seems to be competitive, but more complex effects are also present which cannot be attributed either to prevention of the access of the substrate to the enzyme by the disaccharide or to alterations of the membrane environment. The induction by the sugar of a lag period in the progress curves, and the loss of sensitivity and lag upon storage, are puzzling observations for which no ready answer is available.

The inability of sucrose to inhibit totally the enzymic activity could be explained by the presence in milk fat globule membranes of at least two populations of enzymes with alkaline phosphomonoesterase activity [14] and different sensitivity to the sugar.

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