Mare lactotransferrin: purification, analysis and N-terminal sequence determination

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Mare lactotransferrin has been purified and analyzed. Its molecular mass is 81 kDa. A 28 amino acid long N-terminal sequence was established and a first series of comparisons with other transferrins was performed.

Lactotransferrin *Transferrin* *Mare* *N-terminal sequence*

1. INTRODUCTION

Present knowledge concerning lactotransferrins remains limited: indeed, only human lactotransferrin has been submitted to extensive molecular studies devoted to its complete amino acid sequence [1,2] and to the primary structure of the glycans linked to the polypeptide chain through two N-glycosidic bonds [3] as well as to biological studies reviewed in [4]: thus molecular and evolutionary comparisons with human serum transferrin and hen ovotransferrin could be performed [5]. Only the purification of bovine [6] and rabbit [7] milk lactotransferrins has been further reported. Lactotransferrin was claimed to act to deprive the gut flora of the free iron required for many common gut-associated pathogens [8]. Lysozyme (EC 3.2.1.17) characterized in human but not in bovine milk also represents effective, however non-specific, antimicrobial activities: the mechanism of its role in the defence of the gut from pathogens, yet to be fully explained, is probably intricately interrelated with other components such as lactotransferrin [9–11]. It was thus tempting to develop the study concerning these two components present in various milks. Mare's milk contains simultaneously lactotransferrin and lysozyme. The latter has already been isolated and partially characterized [12]. Here, we deal with the purification, analysis and N-terminal sequence determination of mare lactotransferrin.

2. MATERIALS AND METHODS

Mare's milk was obtained from E. Wettstein, Fohlenhof, Wermatswil, Switzerland. The milk was stored frozen. Heparin–Sepharose CL-6B, Sephadex G-100 and the Mono SHR 5/5 column (5 × 0.5 cm) were from Pharmacia. All other reagents (analytical grade) were purchased from Fluka or Merck, except those employed for the Sequencer which were obtained from Pierce or S.D.S. (Marseilles).

2.1. *Preparation of whey proteins*

The milk was thawed at 4°C, centrifuged at 10000 × *g* for 30 min and after removal of the cream, the skim milk was adjusted to pH 4.7 with 4 M HCl; the caseins were precipitated by heating the milk to 40°C for 30 min. After another centrifugation at 20000 × *g* for 30 min, the whey was decanted and dialyzed against a 0.005 M veronal–HCl, 0.05 M NaCl, pH 7.4 buffer before being applied to a column of heparin–Sepharose.

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2.2. *Polyacrylamide gel electrophoresis*

Sodium dodecylsulfate-polyacrylamide (10%) gel electrophoresis (SDS–PAGE) was performed according to [13] at pH 8.9 in the presence or absence of 1% dithiothreitol; protein bands were stained with Coomassie brilliant blue R-250.

2.3. *Analytical procedures and N-terminal sequence determination*

The amino acid composition was determined after total hydrolysis (5.6 M HCl, containing 1/2000 2-mercaptoethanol; 18, 48 h; under vacuum) with a Biotronik LC 6000 Autoanalyzer. Cystine was determined as *S*-carboxymethylcysteine after reduction and alkylation and tryptophan according to [14]. Automated Edman degradation was carried out in a Beckman 890 C Sequencer, by the 1 M quadrol method in the presence of polybrene [15]. The phenylthiohydantoin amino acids were identified by thin layer [16] and with a Waters Associates ALC/GPC 204 high-pressure liquid chromatograph equipped with a 680 programmer, using a μ-Bondapak C18 column (30 × 0.39 cm) [17]. A Radial-Pak μ-Bondapak C18 column (10 × 0.8 cm) was employed to separate methionine from valine, arginine from glutamic acid and histidine from leucine using the following system: solvent A was 0.02 M sodium acetate (pH 6.3)–methanol (9:1, v/v) and solvent B ethanol 95°; a gradient (curve 8) from 5 to 30% of solvent B over 16 min was followed by isocratic elution with 30% solvent B over 31 min at room temperature. The sugars were determined according to [18] using a Varian Model 3700 gas chromatograph.

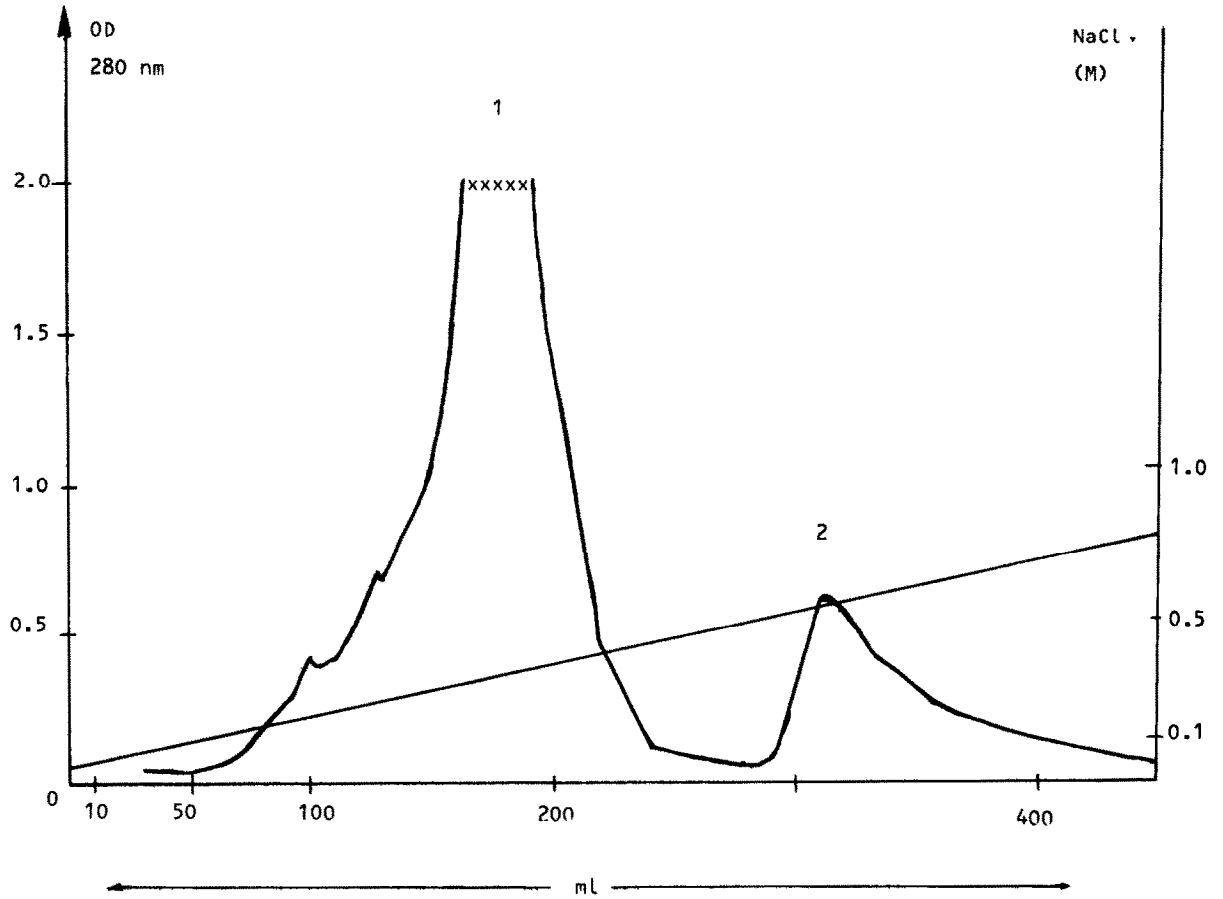

Fig.1. Heparin–Sepharose (25 × 2 cm) chromatography of 250 ml mare whey in 0.005 M veronal–HCl (pH 7.4), 0.05 M NaCl: only the peaks eluted by a linear 0.05–1.0 M NaCl gradient are shown. Flow rate, 10 ml/h.

3. RESULTS AND DISCUSSION

3.1. *Purification and molecular mass*

The complete purification of mare lactotransferrin was achieved by a 2-step procedure. The whey proteins were first submitted to a heparin–Sepharose chromatography according to [19] and [20]. Lactotransferrin and lysozyme were contained in the second peak eluted by the NaCl gradient (fig.1). Dialysis prior to Sephadex G-100 filtration was done in Spectrapor dialysis tubing with an ~15000 Da cut-off: a large part of lysozyme was thus eliminated. The purity of mare lactotransferrin obtained after Sephadex G-100 filtration (fig.2) was proven: (a) by FPLC-chromatography (Pharmacia) (10 mM piperazine-*N,N'*-bis-2-ethanesulfonic acid (PIPES) buffer, pH 6, with a linear 0–0.5 M NaCl gradient): a symmetrical peak was obtained; (b) by determination of a unique N-terminal sequence (see below); (c) by SDS–PAGE: a unique band was characterized corresponding to a molecular mass of 81 kDa, in good agreement with the molecular masses of human lactotransferrin and other transferrins [2,5–7].

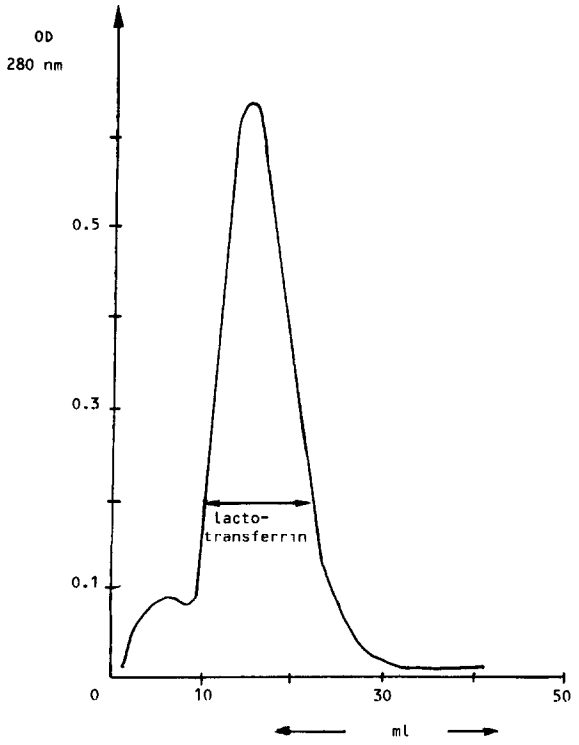

Fig.2. Sephadex G-100 (100 × 1 cm) filtration of the second peak eluted by the NaCl gradient of the heparin–Sepharose chromatography (fig.1). Eluent: 0.1 M sodium acetate (pH 6.5). Flow rate, 10 ml/h.

3.2. *Amino acid and sugar analyses*

The amino acid composition of mare lactotransferrin, compared to the proteins of human and bovine origins, is indicated in table 1. The following sugars were characterized (molar ratios in parentheses): Gal (4), Man (6), GalNAc (1.5), GlcNAc (9.5), NeuAc (1.5). A small quantity of GalNAc was also found in bovine lactotransferrin [6] whereas the presence of this sugar was not reported in the glycans determined in human lactotransferrin [3].

3.3. *N-terminal sequence determination and comparison with other transferrins*

A 28 amino acid long N-terminal sequence of mare lactotransferrin was established (table 2). When only the first 12 N-terminal amino acids of the so far studied transferrins were compared, the

Table 1

Amino acid composition (residues per molecule) of mare lactotransferrin: average values of 5 analyses after different time intervals. Comparison with human (according to the complete structure [2]) and bovine [23] lactotransferrins

Amino acid	Lactotransferrin		
	Mare	Human	Bovine
Asp	72	71	71
Thr	32	31	39
Ser	48	50	45
Glu	70	70	73
Pro	33	35	31
Gly	50	56	43
Ala	71	63	59
Cys	38	32	28
Met	3	6	4
Val	44	49	43
Ile	14	16	17
Leu	60	61	61
Tyr	20	20	19
Phe	25	31	25
Trp	12	11	9
Lys	45	46	42
His	10	9	10
Arg	33	46	32

Table 2

N-terminal sequence of mare lactotransferrin: comparison with human lactotransferrin [2], human serum transferrin [21] and hen ovotransferrin [22]

	1	5	10	15	20	25
Mare lactotransferrin	A P R K S V	– R W C T I S	P A E A A K C	A K F Q R N M K K		
Human lactotransferrin	G R R R R S V	– Q W C A V S	Q P E A T K C	F Q W Q R N M R K		
Human serum transferrin	V P D K T V	– R W C A V S	E H E A T K C	Q S F R D H M K S		
Hen ovotransferrin	A P P K S V	I R W C T I S	S P E E K K C	N N L R D L T Q Q		

The one-letter amino acid abbreviation system is used. Gaps have been placed to maximize the homology. Boxed areas contain residues that are identical (□) or homologous (⬚)

mare protein presented a more pronounced homology with hen ovotransferrin than with human lactotransferrin: the cluster of arginine residues was no longer present. However, when the whole of the so far determined N-terminal sequence is considered, it presents an almost identical homology with the other transferrins.

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