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IDENTIFICATION, PARTIAL PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF γ-GLUTAMYLTRANSPEPTIDASE PRESENT AS A MEMBRANE COMPONENT IN SKIMMED MILK AND MILK FAT-GLOBULE MEMBRANES, AND IN MAMMARY-TUMOUR VIRUS FROM THE MILK OF INFECTED MICE

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Summary

The enzyme γ-glutamyltranspeptidase was reproducibly found to be associated with mouse milk particles; it is present in milk fat-globule membranes and mouse mammary-tumour virus of infected Swiss mice, also in particles from the milk of uninfected mice.

The enzymatic activities observed range among the highest reported for mammalian tissues.

The enzyme was partially purified from mouse mammary-tumour virus, and from milk fat-globule membranes. The molecule requires the presence of detergents to remain soluble, behaves as a high molecular weight component, properties characterizing integral membrane proteins.

Kinetics, and the effect of competitors as well as of specific inhibitors show this enzyme to be identical to the well-known kidney γ-glutamyltranspeptidase ((γ-glutamyl)-peptide:amino-acid γ-glutamyltransferase, EC 2.3.2.2).

Other oncornaviruses budding from cultured cells originally expressing the enzyme in their plasma membrane also incorporate the enzyme in their structure.

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A short report on the presence of enzymatic activity in mouse mammary-tumour virus was presented at the 10th Meeting of the European Tumour Virus Group, Grindelwald, Switzerland, 3—7 October 1976, p. 60.

Introduction

In a previous publication [1] we compared the components of mouse mammary-tumour virus and milk fat-globule membranes, two structures produced by exocytosis in mammary glands of mice. When looking for the presence of peptidases in these structures, we found that L-γ-glutamyl-*p*-nitroanilide, used for testing L-γ-glutamyltranspeptidase [2], was an excellent substrate. This enzyme, discovered by Hanes et al. [3] is a membrane-bound enzyme, widely distributed in higher organisms. Typically found in secretory cells, it appeared to be involved in secretion [4] and also in amino acid and peptide reabsorption by the kidney [5,6].

This study demonstrates the presence of tightly bound transpeptidase in milk particles. Partial purification of the enzyme from mammary-tumour virus and from milk fat-globule membranes is described, and its identity with the well-known kidney enzyme ((γ-glutamyl)-peptide:amino-acid γ-glutamyltransferase, EC 2.3.2.2) is established from several enzymic criteria.

A limited survey indicates the enzyme to be also present in other Oncornavirinae, confirming the common borrowing of cell-coded proteins in the process of viral maturation.

Materials and Methods

Mice. A strain of Swiss albino mice, raised in our laboratory for 20 years, and the RIII strain from the 'Foundation Curie' (Paris) both infected with the mammary-tumour virus, were used. Two uninfected C57BL inbred strains were also studied.

Viruses. Mouse mammary-tumour virus was prepared from mouse milk diluted with a buffer containing EDTA [1] to which was added 0.3 mg/ml phenylmethylsulfonylfluoride; other viruses were obtained through several sources in Belgium, except Semliki forest virus supplied by Dr. M. Horzinek (Instituut voor Virologie, Rijksuniversiteit, Utrecht). Purity and concentration of viral particles were checked by electron microscopy.

Non-sedimentable material from the milk. We used the upper layer, above the 20% glycerol layer, obtained by centrifugation of the diluted skimmed milk at 96 000 $\times g_{av}$ for 1 h [1].

Membranes. Milk fat-globule membranes were obtained as described earlier [1] and fractionated by isopycnic sucrose gradient centrifugation (20–70 g/100 ml).

Reagents. All chemicals were reagent-grade products. Deoxycholic acid was recrystallized from a reagent-grade sodium salt. Phenylmethylsulfonylfuoride, Triton X-100 (scintillation grade) and fluorescamine were from Serva (Heidelberg) and DEAE-cellulose from Whatman. Concanavalin A-Sepharose, *Aspergillus niger* glucose oxidase, reduced glutathione, dithiothreitol, and α-methylmannoside were purchased from Sigma. [^{14}C]Glycylglycine was purchased from Amersham.

Protein estimation. Protein was estimated by a modification [7] of the method of Lowry and coworkers; lower protein amounts, in the range 0.2–2 μg were estimated as follows: to 50 μl test solution (made 0.1 M in $KHCO_3$) were added, with instant shaking, 25 μl of a 1 mg/ml solution of fluorescamine

in acetone, then 1.95 ml of 0.1 M $KHCO_3$. Fluorescence was measured within 30 min at 480 nm under excitation at 390 nm. Crystalline bovine serum albumin was used for calibration.

Enzymic assays. The activity of γ-glutamyltranspeptidase was assayed at the initial velocity [8]; one enzyme unit releases 1 μmol *p*-nitroaniline/min at 37°C [9]. Catalase was estimated spectrophotometrically [10]. The specific inhibitor α-ketoglutarate-γ-L-glutamylhydrazone was synthesized extemporaneously as described [11].

Detection of transpeptidation. The product of the transpeptidation reaction was detected as follows. In a total volume of 50 μl 20 mM Tris-HCl buffer, pH 8.0, 4.8 mU purified viral enzyme (6 U/mg protein) was incubated for 15 min at 22°C with 4 and 10 mM labelled glycylglycine ($120 \cdot 10^3$ and $48 \cdot 10^3$ dpm) each in the presence of 2 mM γ-glutamyl-*p*-nitroanilide or 4.0 mM reduced glutathione as donors. Controls were carried out omitting the enzyme. The reaction was arrested by immersion in ice. The total reaction mixture, followed by one rince, was transferred stepwise onto Whatman 3 MM paper strips and air dried. Electrophoresis was performed at 40 V/cm for 60 min in a refrigerated collidine buffer of pH 6.5. Paper strips were scanned at 2 cm/min in a Packard radiochromatogram scanner fitted with a disc chart integrator.

Screening of other viruses. Activity was measured as described above, except for the addition of 0.7 mg deoxycholate/ml incubation medium. The limit of detection is $2 \cdot 10^{-5}$ unit, which leads to a sensitivity of 1 mU/mg protein in our conditions. Inhibitory activity was ruled out by subsequent addition of known amounts of purified enzyme. The specificity of the reaction was checked by its inhibition by glutathione as well as by the mixture of 5 mM borate and 5 mM L-serine.

Enzyme purification. The viral preparation (20—30 mg protein) was disrupted and chromatographed according to Parks et al. [12]. When most proteins had been eluted by the NaCl gradient, the enzyme was eluted with 1 mg/ml deoxycholate in 0.5 M NaCl. The active fractions (12 units) were dialysed against 10 mM Tris-HCl, pH 8.0, containing 0.2 mM $MgCl_2$ and 1 mg/ml Triton X-100, then applied onto a short (10 cm × 1 cm^2) DEAE-cellulose column equilibrated with the solution just described. One column volume of the buffer was flown through the column, and elution was carried out with a gradient of NaCl. The enzyme activity appeared at 40 mM NaCl.

As regards the enzyme from milk fat-globule membranes, the first two stages of a described procedure were used [13], followed by separation on concanavalin A-Sepharose (3 ml column) [14].

Gel electrophoresis. Discontinuous gel electrophoresis in 7% polyacrylamide (pH 8.9) was used [15]. Enzymic activity was revealed by dipping gel slices in the solution described above for the estimation of the enzyme, then measuring the *p*-nitroaniline released after adequate periods. A duplicate gel was submitted first to Schiff staining, then to Coomassie staining.

Gel filtration. A column (26.5 × 1.5 cm^2) of Sephadex G-200 in 50 mM Tris-HCl buffer, pH 8.0, 0.2 M NaCl, 0.5 mM dithiothreitol, containing 1 mg/ml Triton X-100, was charged with 370 mU purified enzyme (and catalase as a molecular weight marker) in a total volume of 0.2 ml, and eluted at 1.5 ml/h with the same buffer at 4°C. Enzymes were detected as described above.

Results

Enzymic activity in milk fractions from infected Swiss mice

Enzymic activity was repeatedly absent in the non-sedimentable material from all milks studied.

Milk fat-globule membranes contained a mean activity of 0.74 U/mg protein. Fractionation of the latter membrane preparation by sucrose density gradient furnished major bands at densities 1.09, 1.19 and 1.21 g/cm^3 with respective specific activities of 0.34, 0.37 and 0.40 U/mg protein.

Crude membrane fractions of the skimmed milk, sedimenting through a layer of 20% glycerol [1] displayed a specific activity around 1.3. This preparation can be separated on sucrose gradients into the several fractions described in an earlier publication [1]; all fractions, among which typical viral particles were of density 1.17 g/cm^3, were enzymically active, mostly fraction of density 1.14 g/cm^3. (Table I). Viral particles prepared from the milk of another infected strain of albino mice (R III) exhibited a specific activity of 0.5 U/mg protein (Table IV).

Particle-bound state of the enzyme

In order to test the stability of the enzyme molecule toward solubilization, a subsequent sucrose density fractionation of each sucrose fraction was performed: the major part of the activity was retained, in spite of the prolonged treatment at room temperature (Table I).

The typical viral particles were submitted to a CsCl gradient centrifugation: the specific activity of the viral particles (banding at 1.20 g/cm^3) was not significantly reduced (95% of the activity in the virus before the CsCl gradient).

Enzymic activity in milk fractions from virus-free C57BL mice

When skimmed milk from uninfected C57BL mice was submitted to the same fractionation procedure, fractions were recovered at several densities and appeared as vesiculated structures under the electron microscope; no virus-like

TABLE I

SPECIFIC ACTIVITIES OF γ-GLUTAMYLTRANSPEPTIDASE IN MOUSE SKIMMED MILK MEMBRANES

Sucrose gradient centrifugation was done in a Spinco rotor SW-27 at 25 000 rev./min for 16 h at 20°C. Sucrose densities were measured by refractometry at 25°C. Data are expressed as μmol · min^{-1} · mg^{-1} protein at 37°C.

Sucrose densities (g/cm^3)	Infected Swiss mice Sucrose gradient centrifugation		First sucrose gradient centrifugation	
	First	Second	C57BL No. 1	C57BL No. 2
1.2	0.58	0.26	0.32	0.03
1.14	2.06	2.21	0.72	0.10
1.16	1.28	1.08	0.71	0.35
1.17 *	1.01	0.60	0.64	0.31
1.18	1.85	1.16	0.70	0.31

* This fraction contains typical virus particles [1] in infected mouse milk.

particle was observed at density 1.17 g/cm^3. Enzyme activity is present in each density fraction, but vary with the mouse strain (Table I).

Purification of enzymes

Fig. 1 shows the elution profile of viral proteins and activity from the DEAE-cellulose column; addition of deoxycholate was necessary to elute the enzyme, together with several high molecular weight glycoproteins. The latter were removed by a second DEAE-cellulose chromatography in the presence of Triton X-100. Further fractionation was attempted on concanavalin A-Sepharose, but resulted in loss of specific activity. Table II summarizes the results of the two stage purification of the viral enzyme. In a similar fractionation procedure on another batch of crude virus, a specific activity of 7.65 U/mg protein was obtained.

Results of the purification of the enzyme from milk fat-globule membranes are summarized in Table III. In this case affinity chromatography on concanavalin A-Sepharose resulted in a 3-fold purification, but with a low recovery.

Homogeneity of the enzyme from both sources was tested on 7% polyacrylamide gel electrophoresis at pH 8.9. In the absence of detergents, the whole enzymic activity and glycoproteins deposited on the gels remained at the origin (not shown). In the presence of 1 mg/ml Triton X-100 in samples and gels, activity and glycoprotein component moved together in a single peak of R_F = 0.15—0.20 (Fig. 2). No protein or glycoprotein species contaminating the enzymes could be dissociated in this electrophoretic system. Glucose oxidase (mol. wt. 150 000) used as a standard moved with a R_F of 0.48 in the same experiment.

The present results indicate that in the absence of detergent these transpeptidases are not soluble; when solubilized by Triton, their low mobility suggests a low charge density at that pH and/or, more likely, a high molecular weight.

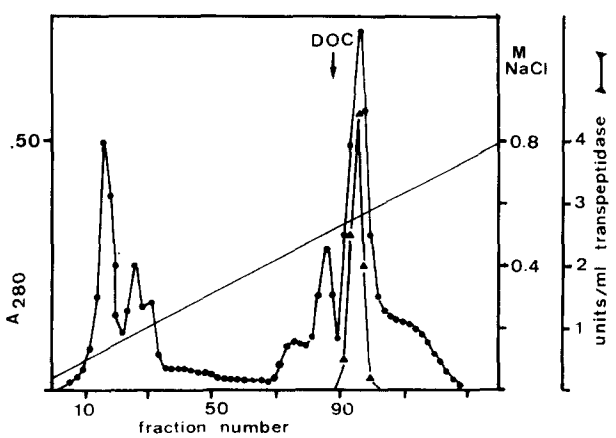

Fig. 1. DEAE-cellulose chromatography of disrupted mouse mammary-tumour virus. ———, the NaCl gradient; ●, the absorbance at 280 nm; ▲, the enzyme concentration in the fractions.

TABLE II

PURIFICATION STEPS FOR VIRAL ENZYME

DEAE, DEAE-cellulose chromatography.

Preparation	Protein (mg)	Total units	Specific activity (units/mg protein)
Disrupted virus	25	18	0.72
Peak of 1st DEAE	6.5	13	2.0
Peak of 2nd DEAE	1.7	10.8	6.0

Molecular characteristics

Molecular weight of the viral enzyme was estimated by gel filtration on Sephadex G-200. The marker catalase (mol. wt. 250 000) appeared as a main peak at K_d = 0.37. The γ-glutamyltranspeptidase was observed in a main peak in the void volume, and in two unresolved minor components at K_d = 0.11 and 0.16. Hence the main species of the enzyme appears to be at least 400 000 in molecular weight.

Biochemical properties of the viral enzyme, and identification with well-known mammalian γ-glutamyltranspeptidases

Transpeptidation of the γ-glutamyl group defines the enzyme as belonging to the class EC 2.3.2.2. Transpeptidation by the viral enzyme involved 11 and 14% of the 10 mM acceptor present with respectively γ-glutamyl-*p*-nitroanilide and glutathione as donors, and 26 and 20% of the 4 mM acceptor present, with γ-glutamyl-*p*-nitroanilide and glutathione, respectively.

The viral enzyme has the following properties:

(1) Expressed as per cent of the activity in the presence of glycylglycine, the activity with L-methionine and glycine as acceptors were 44 and 19%, respectively; corresponding values of 32 and 20% were reported for the kidney enzyme [8].

(2) With 20 mM glycylglycine and 0.5 mM γ-glutamyl-*p*-nitroanilide as substrates, the activity was inhibited to the extent of 50% by 0.375 mM glutathione; one of the kidney enzymes responds similarly [16].

(3) Initial velocities were plotted as the Lineweaver-Burk linear function. The K_m for glycylglycine in the presence of 2.5 mM donor was 9.3 mM (r^2 = 0.99; n = 6), and the K_m for the donor was 0.67 mM in the presence of 50 mM

TABLE III

PURIFICATION STEPS FOR MILK FAT-GLOBULE MEMBRANE ENZYME

Preparation	Protein (mg)	Total units	Specific activity (units/mg protein)
Membranes	119	47	0.40
Deoxycholate extract	12.8	23	1.80
10—70% $(NH_4)_2SO_4$ fraction	5.4	24	7.0
Concanavalin A-Sepharose peak	0.17	3.6	21.2

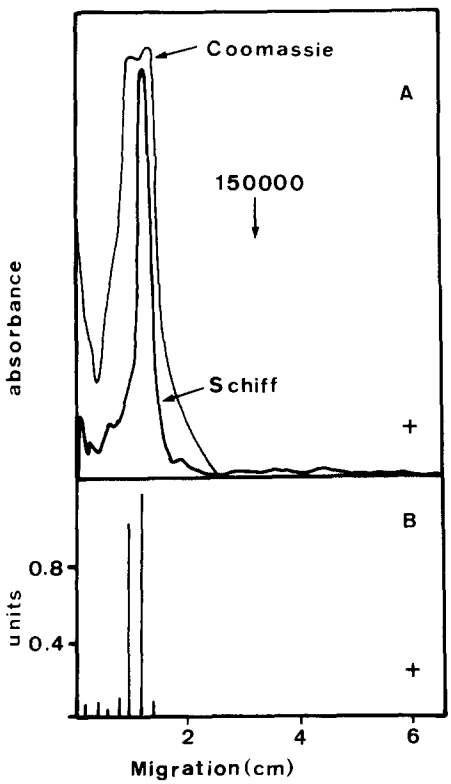

Fig. 2. Discontinuous gel electrophoresis of the native purified viral enzyme in the presence of Triton X-100 at pH 8.9. (A) Schiff and Coomassie stains sequentially applied to the same gel and recorded. (B) Transpeptidase activity in gel slices (1.5 mm) of duplicate gel. Bromophenol blue migrated 6 cm.

glycylglycine ($r^2 = 0.99$; $n = 6$). The latter value is very similar to the values of 0.9 mM and 0.8 mM γ-glutamyl-*p*-nitroanilide characterizing the rat kidney enzyme [8] and the human kidney enzyme [16], respectively.

(4) Our enzyme was fully inhibited by the mixture of 5 mM each of borate and L-serine (not by either alone). A 93% inhibition was reported for the kidney enzyme [17]. Inhibition by this mixture seems to be a unique property of γ-glutamyltranspeptidases.

(5) A 83% inhibition of the viral enzyme occurred in the presence of 2.5 mM α-ketoglutarate-γ-glutamylhydrazone: this synthetic analogue of glutathione also strongly inhibited the kidney enzyme, with a K_i of 0.4 mM [8].

(6) Addition of 2.5 mM iodoacetamide to the assay, or preincubation of the enzyme overnight with this reagent before the assay, resulted in 33% loss of activity. Beef and human kidney enzymes also seem partly and unspecifically inactivated in the presence of SH-reagents [13,16].

The enzyme purified from milk fat-globule membranes has very similar properties.

In summary, the viral and the milk fat-globule membranes enzymes studied do not differ from the several well-characterized kidney γ-glutamyltranspeptidases by any of the main enzymic criteria.

TABLE IV

ACTIVITY OF γ-GLUTAMYL TRANSPEPTIDASE IN SOME VIRUSES

n.d., not done. + and ++, virus concentrations in the range of respectively $1 \cdot 10^{12}$ and $1 \cdot 10^{13}$ particles/ml, as estimated by electron microscopy.

Viruses	Cells on which viruses were grown	Activity at 37°C (mU/mg protein)	Protein (mg/ml)	Virus particle concentration
Oncornavirinae				
(1) C Type				
Avian				
Avian myeloblastosis	Chick fibroblasts	14	1.6	++
Mammalian				
Rauscher leukemia				
Batch 10259	GL SV-5 (infected mouse spleen)	0	0.30	++
Batch 10586	GL SV-5 (infected mouse spleen)	0	0.53	+
Simian sarcoma (NCI)	unknown	60	0.51	+
	NRK (rat kidney)	1700	1.60	++
(2) B Type				
Mouse mammary tumour				
Swiss	milk	600—1200	1.00	++
R III	milk	500	1.00	++
(3) Other				
Mazon-Pfizer monkey	NC 37 (human lymphocytes)	57	3.86	++
Myxoviruses				
Influenza PR8	embryonated egg	0	n.d.	++
Paramyxoviruses				
Newcastle disease	embryonated egg	0	n.d.	++
Herpes viruses				
Bovine infectious rhinotracheitis	MD BK (bovine kidney)	0	0.32	+
Alphaviruses				
Semliki forest,				
Batch a	BHK21 (baby hamster kidney)	0	n.d.	+
Batch b	neuroblastoma line	0	n.d.	+

Presence in other viruses

Examination of several other viruses for the presence of γ-glutamyltranspeptidase was carried out. Table IV shows that several oncornaviruses, all of them budding from the plasma membrane, possess the enzyme, some at very high specific activity.

Discussion

The presence of γ-glutamyltranspeptidase in mammary-cell products has been reported only recently in bovine milk [18,19], and its induction in the cells described during lactation. The present investigation shows that this enzyme is a major and stable component of the membrane surrounding milk-fat globules, of mouse mammary-tumour particles and of vesiculated structures in skimmed milk.

In these structures as in kidney, γ-glutamyltranspeptidase is present at comparatively high specific activities; indeed values 10 to 100 times lower are usually found in other mammalian tissues [20].

The present results show the enzyme to be particle bound, as is the case in kidney [5,13,21,22], liver [23], cerebral capillaries [24] and lymphoid cells [25]; this is in accordance with other studies [1,26] that show the structures studied here to be derived from the plasma membrane of the mammary cell by budding.

In addition to exhibiting enzymic identity with the kidney enzymes our viral and milk fat-globule membranes enzyme share some macromolecular properties with the well-studied kidney enzymes prepared without proteolytic treatment. (a) Membrane isolation results in enzyme enrichment. (b) The enzyme is not extracted at neutral pH by the salts solutions that are used in purifying membranes and viral particles; it can be dissolved by detergents, and possesses a high tendency to reaggregate in their absence [16,17,22] as shown by the necessary addition of detergents throughout the purification. (c) When dissolved in the presence of Triton X-100, it is excluded from Sephadex G-200 [16,17,22,27]. Such properties denoting a high hydrophobicity, eventually restricted to one single domain of the molecule [22] are necessary and sufficient to classify this enzyme as an integral membrane protein [28].

Among viruses tested for the presence of the enzyme, four viruses, all belonging to the oncornavirinae, possess the enzyme: one avian and one simian C type, one B type, and one D type, the Mason-Pfizer virus. The enzyme is lacking in another oncovirus. Further studies may indicate whether γ-glutamyltranspeptidase is restricted to Retroviruses, whether its presence is linked to budding, a defined species of exocytosis, or whether in any case its presence is restricted to cell types in which the viruses replicate. The relation to budding would be supported by the fact that, although the majority of the viruses tested leave the cell by the budding process (Table IV), the enzyme was found only in viruses produced by cell lines or cells in vivo that have been shown to possess the enzyme in the plasma membrane: mammary cells (this investigation and Ref. 1), kidney cells [21], lymphocytes [25].

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