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Purification and characterization of avian glycolipid: β-Galactosyltransferases (GalT-4 and GalT-3): cloning and expression of truncated βGalT-4★✪

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Acidic glycolipid of ganglio-(containing sialic acid) and sialyl-lactofucosyl-type, SA-Le^x (containing sialic acid and fucose) are developmentally regulated and appear to be ubiqitous on neuronal and cancer cell surfaces of animals. Two glycolipid: β-galactosyltransferases, GalT-3 and GalT-4, were characterized in embryonic chicken brain (ECB). Based on substrate competition experiments, these two activities were believed to be due to expression of two gene products. A cDNA fragments (about 600 bp) encoding the catalytic domain of the GalT-4 (UDP-Gal:LcOse3Cer β1,4galactosyltransferase) from ECB and human Colo-204 were isolated. These cDNAs were expressed as a soluble glutathione-S-transferase fusion protein (48 kDa) in *Eschericchia coli*. Interactions between GlcNAc-, UDP-hexanolamine-, and α-lactalbumin were studied with the purified fusion protein (recombinant and truncated). Functionally it was similar to that of native GalT-4 purified (40000-fold) from 11-day-old ECB. GalT-3 (UDP-Gal:G_{M2}β1,3galactosyltransferase) was purified from 19-day-old ECB, and a polyclonal antibody was produced against the peptide backbone for immunoscreening of a λZAP ECB cDNA expression library. Each of the GalT-3 peptides (62 and 65 kDa was analyzed by protein fingerprinting analysis indicating a similar peptide mapping pattern.

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Abbreviations: DSS, detergent solubilized supernatant fraction; ECB, embryonic chicken brains; G_{M1}, Galβ1,3GalNAcβ1,4(α2,3NeuAc) Galβ1,4Glc-ceramide; GSL, glycosphingolipids; GST, glutathione-S-transferase; Lc3, GlcNAcβ1,3Galβ1,4Glc-ceramide; L_{M1}, NeuAcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4Glc-ceramide; SLS, sodium lauryl sarcosinate; TMD, transmembrane domain.

Over the last three decades several (eighteen to twenty) glycolipid:glycosyl transferases (GSL:GLTs) have been characterized from eucaryotic (normal or developing) tissues and cells (normal, rapid growing, or tumors), which catalyze *in vitro* biosynthesis of various well characterized GSLs. Several different glycolipid: glycosyltransferases [1-14] have been characterized from different eucaryotic systems. Each glycosyltransferase is specific according to its donor sugar nucleotide substrate. They catalyze the transfer of specific monosaccharide residues from the sugar nucleotide donor to the non reducing terminus of the growing oligosaccharide chain of an acceptor glycosphingolipid molecule. The specificity of an acceptor molecule may reside on the sugar chain or on both the ceramide moiety and the sugar moiety. The specificity of a glycosyltransferase reaction is characterized by the enzymes of high affinity with respect to both the 'donor' and the acceptor structure. It is believed that the glycosyltransferase specificity for the nucleotide sugar donor substrate is absolute. Specificities concerning the sugar acceptor substrate can vary. Some GSL:GLTs exhibit a broad acceptor specificity, catalyzing transfer of sugars at comparative rates to glycolipids, glycoproteins, or oligosaccharides [6, 8, 11, 13]. Other glycosyltransferases exhibit a stringent requirement regarding the nature of the acceptor and/or the oligosaccharide moiety of the acceptor as will be discussed. The substrate specificities of a transferase catalyzing the same reaction *in vitro* may also vary depending on its tissue or origin [1, 3-5]. Until now most of these specificity studies have been carried out with highly purified glycosyltransferases (GLTs) of tissue- or cell-origin [9, 10]. Studies completed with the recombinant over-expressed truncated GalT-4 proteins will be mentioned in the later sections. Studies with GalT-3 protein will be published elsewhere.

The GSL:GalTs which form the largest family [6, 10] catalyze reactions that utilize UDP-α-galactose as the sugar donor and potent glycolipid acceptors. The galactosyltransferases that catalyze different positional and anomeric linkage formations in various GSLs comprise family of more than seven individual gene products [9]. Most of these activities were characterized and purified from animal cells. The putative amino-acid sequence from cDNA sequences of at least three GSL:GalT is reported and will be discussed in the following sections. Biosynthesis of two GSLs, SA-Le^x and G_{M1} of lacto- and ganglio-families, respectively (Fig. 1), are catalyzed by two specific β-galactosyltransferase proteins (Fig. 2).

Among the galactosyltransferases, a milk β1-4GalT (lactose synthetase A protein, EC 2.4.1.22) is capable of using glucose as an acceptor in the presence of α-lactalbumin (lactose synthetase B protein) leading to the biosynthesis of lactose [15-17]. During the past

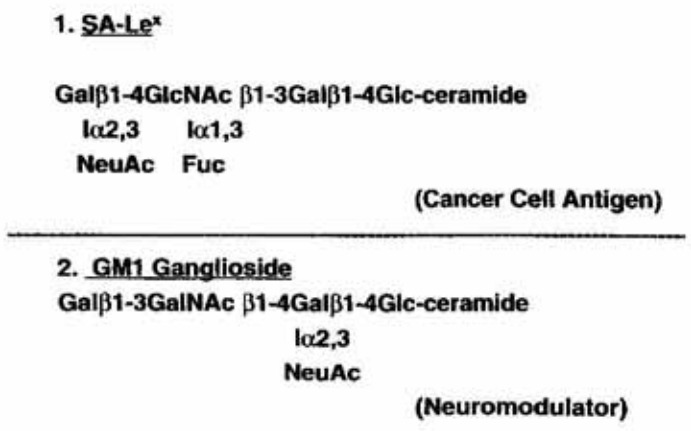


Figure 1. Structure of sialic- and fucose-containing biologically active two glycosphingolipids.

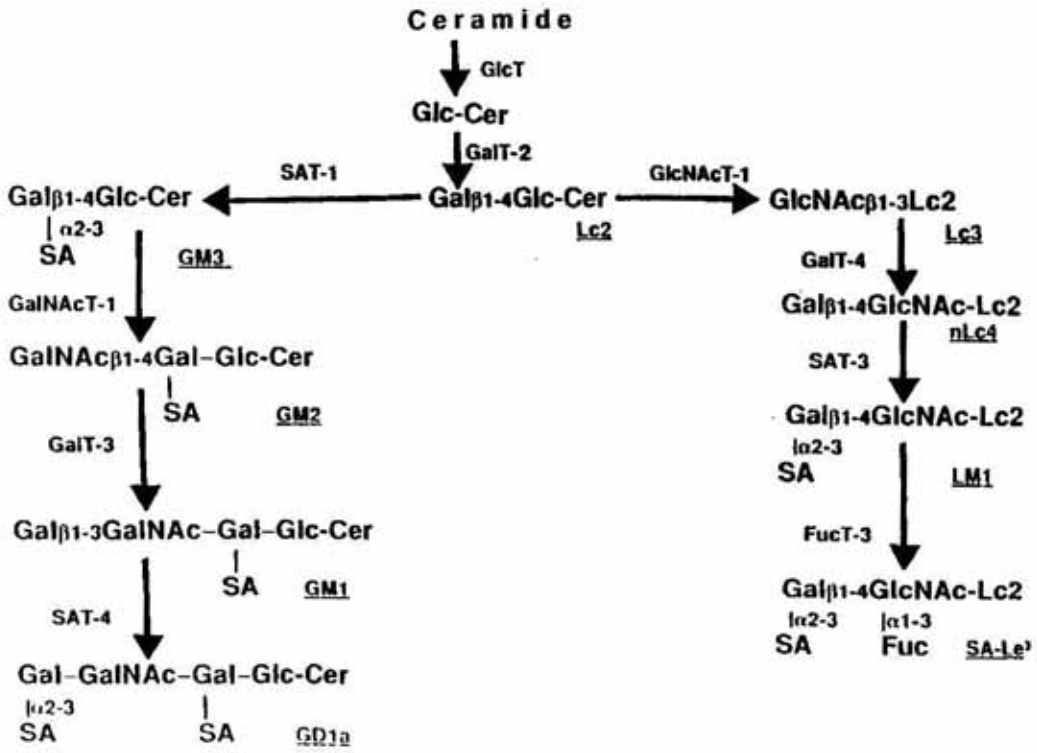


Figure 2. Proposed pathway for biosynthesis of glycosphingolipids of ganglio- and lacto-families.

two decades the homologous protein 'A', catalyzing transfer of galactose to glycolipid (without modulation by α-lactalbumin), has been characterized and isolated from rabbit bone marrow [18, 19], sera [20–22], embryonic chicken brain [5, 7, 23–26], rat prostate tumor [27, 28], neuroblastoma [3, 29, 30], human colon carcinoma [31–33], adenocarcinoma [34, 35], and mouse T-lymphoma [36]. In addition to milk A protein [15–17], the GalT-4 from mouse T-lymphomas (104000-fold) and embryonic chicken brain (40000-fold) [24, 37, 38] have been purified to homogeneity and polyclonal antibodies against ECB-GalT-4 have been produced [24] to study distribution at subcellular organelles and cloning work. Kinetic properties of both GalT-4 (104000-fold) and GalT-3 (5000-fold) have been studied in detail with enzymes purified from T-lymphomas [37] and ECB [23, 24], respectively. The ability of the purified (40000-fold) GalT-4 from ECB [24] and T-lymphomas to transfer galactose to glycosphingolipids (LcOse3Cer or GlcNAcnLcOse4Cer) (Fig. 2), glycoproteins (SA^-, Gal^-, α_1 AGP) and free oligosaccharides has been tested.

A 1.3 kb cDNA clone coding for bovine galactosyltransferases was first isolated from a λgt11 expression library by immunological screening with monospecific polyclonal antisera to the affinity purified bovine milk GalT [39]. The nucleotide sequence predicted an open reading frame (ORF) which codes for 334 amino acids with a M_r 37645 (Fig. 3). Based on the M_r 57000 for the membrane-bound enzyme, the clone accounted for 61% of the coding sequences from the NH_2-terminal end of the protein. A 1.7 kb insert was used from λgt10 human liver cDNA library for human β1-4GalT, the sequencing of which revealed a 783 bp galactosyltransferase coding sequence, with the remainder of the sequence corresponding to the regions of the mRNA downstream from the termination codon. The homology at the amino acid level was 80%, and 91% to the partial sequences of bovine and human milk β1-4GalT, respectively [40]. A full length murine β1-4GalT was cloned from a

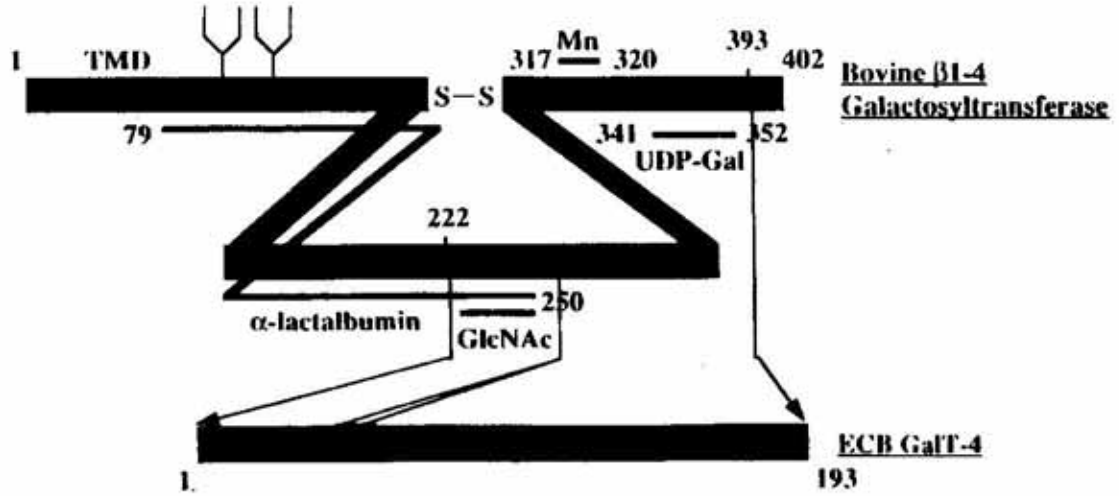


Figure 3. A schematic representation of the putative structural and functional domains of β1-4galactosyltransferase (GalT-4).

Top bar drawing: bovine β-galactosyltransferases drawn from the published sequence data [39]. Bottom bar graph is drawn from embryonic chicken brain GalT-4 enzyme sequence data published from our laboratory [44-46].

murine cDNA library using a bovine β1-4GalT probe. The sequence of a full length murine cDNA β1-4GalT clone, an ORF of 399 amino acids, revealed two sets of starts sites for transcriptional initiation [41, 42]. No apparent sequence similarity was detected between bovine β1-4GalT and α1-3GalT (GalT-5 described below) which was cloned by immunological screening of a bovine λgt11 library [43]. However, both proteins were found to contain a cysteine residue (Cys-298 in α1-3GalT and Cys-339 in β1-4GalT), followed at a distance of 5 to 6 amino-acid residues by a hexapeptide with the sequence B-Asp-Lys-Lys Asn-A(A = Glu/Asp; B = Arg/Lys). The hexapeptide is also converted in murine and human GalTs.

According to the differential trace acetylation experiment reported recently [47], the binding of α-lactalbumin to β1-4GalT (galactosyltransferase) results in the decrease in reactivities of lysine 93 and 181 and increased the reactivities of one or more of lysines 230, 237, and 241. On the basis of this observation, it was proposed that the NH_2-terminal region of bovine β1-4GalT between residue 79 and around 250 is involved in the interaction with α-lactalbumin, and, by implication, with acceptor Glc or GlcNAc.

An 600 bp ECB cDNA fragment, (truncated GalT-4) a homologue [24, 44, 45], to mammalian β1-4GalT, has been expressed in *E. coli*, as GST fusion protein [46]. The expressed GST-GalT-4 (48 kDa) has been found to be catalytically active [47] with similar substrate specificity to that of native ECB. Purified GalT-4 catalyzed *in vitro* biosynthesis of nLcOse4Cer (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-ceramide) from LcOse3Cer (GlcNAcβ1-3Galβ1-4Glc-ceramide). Western blot analysis of the fusion protein showed positive immunoreactivity with antibody raised against bovine β1-4GT [46, 47].

Biosynthesis of G_{M1} ganglioside (Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc-ceramide) from G_{M2} ganglioside (GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc-ceramide) is catalyzed by a β1-3galactosyltransferase, GalT-3 first characterized in embryonic chicken brains (13- to 19-day-old) [6, 48] (Fig. 2). The enzyme activity was subsequently detected in frog and rat brains [49], chick neural retinal cells [50], chick embryo liver [51], and rat liver [52]. Compared to β1-4GalT, the GalT-3 activity level is much lower in cultured cells derived from Tay-Sachs diseased patients (TSD) [53]. In the embryonic chicken brain (ECB) GalT-3

is developmentally regulated [5, 23, 24] and is especially enriched in 19-day-old brains. During solubilization from the ECB Golgi-rich membranes, a β1-4galactosyltransferase, GalT-4 (Fig. 2) was co-solubilized and separated [26]. A high titer polyclonal antibody against highly purified ECB GalT-3 has been produced [23, 24] for further cloning purposes [54]. Kinetic studies and immunological characterization of GalT-3 from 19-day-old ECB has been reported [23, 24].

Purified GalT-3 demonstrates a low K_m for both donor (UDP-galactose) and acceptor (G_{M2}) substrates [23, 24]. Substrate specificity studies with the purified enzyme clearly show the absence of any other glycosyltransferase activities. It is also noteworthy that GalT-3 exhibits a very stringent specificity for acceptor structure; thus, the enzyme is not active on free *N*-acetylgalactosamine (GalNAc) or pNP-GalNAc. This property is in marked contrast to that found for GalT-4 discussed below [55]. Also the potential glycoprotein substrate, ovine sub-maxillary asialo-mucin, is not an acceptor for GalT-3 under the published experimental conditions but inhibits the transfer of galactose to G_{M2} ganglioside in a concentration-dependent manner.

The specificity of GalT-3 for G_{M2} and GalT-4 for Lc3 have been analyzed for the contribution of acceptor substrate structure on GalT-3 and GalT-4 activities. Modified glycosphingolipids, either completely deacylated (lyso-G_{M2}, lyso-Lc3) or, with the long chain (C16–C18) fatty acid in the ceramide moiety replaced by an acetyl (C2) group (acetyl-G_{M2}, acetyl-Lc3), were chemically synthesized. These compounds, which differ from one another with respect to structure as well as hydrophobicities, are tested as potential substrates for GalT-3 and GalT-4. A comparison of the kinetic parameters of lyso-, acetyl-G_{M2} and natural G_{M2} with GalT-3 as enzyme source indicates that both the K_m and V_{max} values are unfavorably changed (10-fold increase in K_m, 4- to 8-fold decrease in V_{max}) toward the modified substrates [24, 56]. This clearly shows that, besides its specificity for the *N*-acetylgalactosaminyl acceptor terminus, GalT-3 protein also has affinity for the acceptor (G_{M2}) structure provided by the ceramide moiety. Whether this requirement is specific for a particular structure or arrangement or is more of a general requirement for hydrophobicity (where the effect of the ceramide fatty acid may be replaced by hydrophobic amino-acid residues of glycoprotein around an O-glycosylation site) remains to be seen with the cloned and expressed enzymes (will be published elsewhere).

MATERIALS AND METHODS

Materials

Tissues and reagents were obtained from the commercial sources as indicated below.

Tissues. Fertile chicken eggs, Creighton Brothers (Warsaw, IN) and Hobart Hatchery (South Bend, IN) whole rabbit blood, Hiatt's Garden Farms (Rochester, IN) bovine blood, local slaughterhouse; bovine brain, Martin's Custom Butchery (Wakarusa, IN).

Proteins, enzymes and antibodies. Bovine serum albumin (Fraction V), α-lactalbumin, plasminogen, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, Sigma Chemical Co. (St. Louis, MO), SDS/PAGE molecular weight standards, Sigma Chemical Co. (St. Louis, MO) and Novex (San Diego, CA). Avidin D Horseradish *Eco*R1, *Xho*1, *Sna*B 1, *Not*1, *Sac*1, *Thermus aquaticus* (Taq) polymerase, T4 ligase, Promega Co. (Madison, WI) cloned pfu DNA polymerase, Stratagene, CA.

Detergents. Sodium taurodeoxycholate, sodium lauryl sarcosinate (SLS), sodium taurocholate, sodium cholate, Sigma Chemical Co. (St. Louis, MO); Tween 20, Cutscum, Triton X-100, Triton CF-54, Fisher Scientific company (Fairlawn, NJ); Zwittergent 3-14, Calbiochem (San Diego, CA).

Glycosphingolipids. All gangliosides and glycosphingolipids were isolated in our laboratory from bovine brain and rabbit red blood cells, following previously described methods [57]. Bovine brain ceramide was purchased from Sigma Chemical Co. (St. Louis, MO).

Autoradiographic supplies. X-OMAT AR diagnostic film, GBX fixer and replenisher, and developer and replenisher, Eastman Kodak Co. (Rochester, NY); Medical X-Ray AIF RX film (Fuji, Japan).

Blotting membranes. BA 83 nitrocellulose, Schleicher and Schuell (Keene, NH); Immobilon-P (PVDF), Millipore Corporation (Bedford, MA).

Kits and Vectors. PCR amplification kit, Perkin Elmer Cetus (Norwalk, CT); Sequenase Version 2.0 DNA sequencing kit, United States Biochemicals Corp. (Cleveland, OH); Probe Eze random prime DNA labeling kit, 5'-3' Inc. Boulder, CO; QIAEX Gel extraction kit, ZIAGEN (Chatsworth, CA), pBK-CMV, Stratagene; modified pGEX-3AKT was a gift sample from Dr. Robert Bell (Duke University, Durham, NC).

Methods

Detergent solubilization of Golgi-enriched membranes. Both GalT-3 and GalT-4 were solubilized from their membrane bound forms by the use of a neutral detergent, Cutscum. The optimal protein:detergent ratio for the solubilization of GalT-3 from 19-day-ECB was determined to be 0.8:1.0, while GalT-4 was optimally solubilized at a protein:detergent ratio of 2.0:1.0 from 11-day-ECB [32, 55]. The homogenized membrane preparation was diluted with HEMSBIN buffer (Hepes, 20 mM, pH 7.0; EDTA, 1.0 mM; 2-mercaptoethanol, 0.15; soybean-trypsin inhibitor, 0.005%; benzamidine, 5 mM; sucrose, 0.32 M), if required, to maintain the optimal protein:detergent ratio and to keep the final detergent concentration from exceeding 1.0%. Cutscum was added directly to the homogenized buffy coat and stirred at 4°C for 1 h. The mixture was then centrifuged at 100000 × ***g*** for 1 h at 4°C. The supernatant thus obtained was named „detergent solubilized supernatant (DSS)" and stored in aliquots of varying size. Galactosyltransferase activity in the DSS was stable for more than 12 months at -20°C [58].

Assay of galactosyltransferase activities. Standard glactosyltransferase reaction mixtures contained the following components (in micromoles unless otherwise stated) in a 0.04-0.06 mL incubation volume: acceptor, 0.005-0.05; Cutscum 0.02 to 0.2 mg; $MnCl_2$, 0.25; cacodylate/HCl buffer, pH 7.0, 10.0; UDP-[^{3}H]Gal, 0.001-0.01 (2-3 × 10^6c.p.m. $\mu mole^{-1}$), and enzyme fractions containing 0.01-0.02 mg protein (either GST-GalT-4 or GalT-3 purified protein). The mixtures were incubated at 37°C usually for 1 to 2 h (during which the rates of the glactosyltransferase reactions were constant with time) after which the reaction was stopped by the addition of 0.01 ml of 0.025 M EDTA. The reaction mixtures were spotted on Whatman 3MM chromatography paper and subjected either to high-voltage electrophoresis (2000 volts, 1 h) or descending chromatography in 1% sodium borate, pH 9.0 [6]. In either case, the radioactive reaction product remained at the origin while the unreacted radioactive nucleotide sugar and its degradation products were removed. The origin as well as 1-inch squares above and below the origin were cut from the dried chromotography paper, and the radioactivity was determined by a toluene-based liquid scintillation system using a Beckman model LS-3801 scintillation counter.

Q-Sepharose column chromatography. Q-Sepharose (Pharmacia) was washed well with deionized water and equilibrated with 10 vol. of equilibration buffer (20 mM Bis/Tris; pH 6.5, containing 1 mM EDTA and 3 mM β-mercaptoethanol). Partially purified fractions containing GalT-3 and GalT-4 activities were dialyzed against equilibration buffer and applied onto a Q-Sepharose (strong anion exchange) column at ratio of 10 to 15 mg protein/ml of gel. The column was maintained at

a flow rate of 1 drop/10 s. The effluent was recycled twice. The column was washed with 10 column volumes of equilibration buffer until no protein was detected. GalT-3 was eluted from the column in a batchwise manner using a gradient of NaCl ranging from 0.1 to 1.0 M in Q-Sepharose equilibration buffer. Protein bands were visualized on SDS/PAGE by silver staining [59] and Western-immunoblotted against anti-GalT-3 antibody [24].

Protein fingerprinting of purified GalT-3. Protein fingerprinting [60] can serve as a powerful tool for determining if proteins are related. This method utilizes partial proteolytic digestion of proteins followed by SDS/PAGE separation of peptide fragments to generate a „fingerprint" that is characteristic of the particular protein substrate and protease used for cleavage. The degree of similarity in the peptide fingerprinting pattern of two proteins at the amino-acid sequence level indicating a common genetic/or evolutionary origin.

One hundred microliters of purified embryonic chicken brain GalT-3 (about 2 μg protein) was resolved on a preparative 10%, 0.75 mm thick SDS/PAGE minigel at constant voltage (70 volts). Following electrophoresis, the gel was stained for 20 min with Coomassie stain (0.25% Coomassie brilliant blue in 20% isopropanol and 7% glacial acetic acid) followed by destaining in 5% acetic acid. Total destaining time was restricted to less than 2 h. The two protein bands were visualized, excised, and the gel slices containing peptide samples were overlaid with an 'overlay solution' (0.125 M Tris/HCl, pH 6.8, 0.1% SDS, 3% β-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue) on a second gel (18%). The protease TPCK-trypsin (0.1–0.2 μg) was dissolved in its appropriate reaction buffer (2-fold diluted with 50% glycerol, 0.025% bromophenol blue), and loaded in a volume of 5 μl over the peptide sample in the well. Electrophoresis was started at 70 volts until the dye band migrated at the interface between stacking and separating gels. At this time the electrophoresis was interrupted and the digestion was allowed to proceed for 1 h at room temperature. The peptide fragments were subsequently resolved on the 18% separating gel by electrophoresis at 200 volts. Following electrophoresis, the peptides were fixed on the gel (40% methanol and 10% acetic acid) and stained by the silver-staining protocol of Merril *et al.* [59].

GSH-agarose column chromatography. The bacterial cell lysate (20 ml) was loaded on to 2 ml glutathione-agarose resin pre-equilibrated with 10% PBS containing 0.5% Triton X-100. After recycling the effluent three times, the column was washed with 10% PBS containing 1% (w/v) Triton X-100, and 10 mM $MgCl_2$. Bound fusion protein was eluted with 25 mM Hepes, pH 8.0, containing 5 mM reduced glutathione, 1 mM dithiothreitol, 10 mM $MnCl_2$, 0.1% (w/v) Triton X-100, and 10% (v/v) ethylene glycol. The elution profile of the GST-fusion proteins from the glutathione-agarose column was monitored by assay for glutathione-S-transferase [61] and/or GalT-4 activities.

Solid-phase binding assay. This assay has been designated to study the interaction between GalT-4 and α-lactalbumin. Alpha-lactalbumin (0 to 100 μg dissolved in a bicarbonate buffer, 0.1 M containing 0.5 M NaCl, pH 8) was loaded onto the wells of a 96-well titer plate and allowed to coat the well overnight at room temperature. Remaining areas in the wells were blocked with 0.5% (w/v) gelatin, in phosphate-buffered saline (PBS). GST-GalT-4 fusion protein was added and incubated with α-lactalbumin in the presence of Mn^{2+} (5 mM) and GlcNAc (5 mM) at 37°C for 1 h. The plates were washed with washing buffer (PBS containing 0.1% (w/v) Tween-20, Mn^{2+} 5 mM, and GlcNAc 5 mM), and then incubated with anti-GST polyclonal rabbit antiserum (1:200 dilution in the washing buffer) for 2 h at room temperature. Excess primary antibody was washed off and the plate was subsequently incubated with sheep anti rabbit-IgG-peroxidase conjugate at 1:2000 di-

lution in the washing buffer. *O*-Phenylenediamine was used as chromogen. The absorbance of the colored product was quantitatively determined at 405 nm on an ELISA plate reader (Titertek Multiskan Plus).

Glutathione S-transferase assay. Spectrophotometric assay for glutathione *S*-transferase activity was performed as described by Habib & Jacoby [61]. The enzyme catalyzes the conjugation of DNB (dinitro-benzene) with glutathione and results in a DNB-glutathione product with a strong molar absorptivity at 340 nm (ε = 9.6 $mM^{-1}cm^{-1}$). The assay was done in UV-transparent cuvette in a final volume of 500 μl. The reaction mixture contained 100 mM potassium phosphate buffer, pH 6.5, 1 mM 1-chloro-2,4-dinitro-benzene (CDNB), and 1 mM reduced glutathione. The reaction was started by adding 5 to 50 μl of enzyme fraction. Change in absorbance at 340 nm was continuously measured for a period of 3 to 5 min in Response Spectrophotometer from Gilford Systems.

α-Lactalbumin-Sepharose column chromatography. α-Lactalbumin was coupled to cyanogen bromide-activated Sepharose 4B (9–10 mg of α-lactalbumin/ ml of Sepharose). The coupled gel was equilibrated in 10 vol. equilibration buffer (20 mM Hepes, pH 7.0; 0.1% (v/v) β-mercaptoethanol; 0.1% (w/v) Triton X-100; 20 mM $MnCl_2$; 20 mM *N*-acetylglucosamine; 0.1 M NaCl). The bacterial cell lysate (expressing GST-GalT-4) (10–20 mg protein) of an aliquot (1–2 ml) of GSH-agarose eluent (100–300 μg of protein) was diluted with an equal volume of 2-fold concentrated equilibration buffer and loaded onto the column (1–2 ml of gel), and the effluent collected at a flow rate of 1 drop per 10–20 s. The effluent was recycled once. The column was washed with 10–15 column volumes of equilibration buffer until no protein could be detected. Elution of enzyme activity was accomplished with elution buffer (equilibration buffer containing 20 mM EDTA, but lacking $MnCl_2$ and *N*-acetylglucosamine). The eluents from the affinity columns were analyzed for GST and GalT-4 activities. Aliquots of the eluents were precipitated with 10% trichloroacetic acid for SDS/PAGE and Western blot analysis.

RESULTS AND DISCUSSION

We have expressed an 600 bp ECB cDNA fragment, homologous to mammalian β1,4-galactosyltransferases, in *E. coli* as GST fusion protein (48 kDa). In order to get the maximum expression of the fusion protein in soluble form, we allowed the bacterial culture to grow at room temperature during the post-induction period. This modification of the expression condition doubled the amount the soluble fusion protein obtained after GSH-agarose chromatography [46]. The expressed fusion protein was also catalytically active, with substrate specificity similar to that of native ECB GalT-4 (catalyzes biosynthesis of nLcOse4Cer from LcOse3Cer) [18, 24, 26]. The GST-GalT-4's affinity for the acceptor and donor substrates and Mn^{2+} is comparable to that of the ECB native GalT-4 [24], although its specific activity is several folds lower than that observed in the case of native GalT-4. Perhaps addition of the fusion protein has an effect on the k_{cat} of the reaction catalyzed by truncated protein. The fusion protein was also found to be immunoreactive, on Western blot analysis ([47]; will be published elsewhere), with antibody raised against the purified ECB GalT-4; this antibody also inhibited the catalytic property of GST-GalT-4. This observation strongly indicates that the about 600 bp cDNA codes for the ECB GalT-4. Since the 600 bp cDNA bears approx. 70% homology to other β1,4-galactosyltransferases that efficiently catalyze galactosylation of N-linked glycoproteins, it appears that this enzyme is involved in transferring galactose to both glycosphingolipid and N-linked glycoprotein. The conclusion is also consistent with the biochemical and immunological characteristic of β1,4- galactosyltransferases including one from ECB [24].

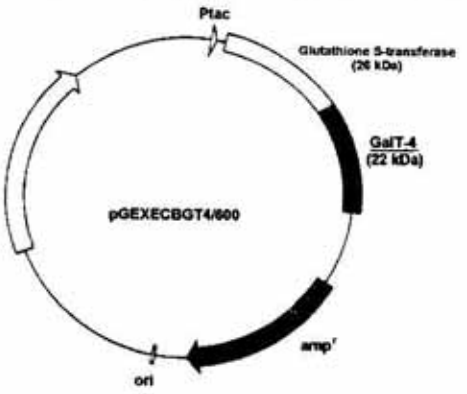


Figure 4. Vector construct for expression of GST-GalT-4 fusion protein.

A schematic diagram of the plasmid vector, pGEX-ECBGT4/600, bearing the cDNA fragment (about 600 bp) of ECB GalT-4 is represented.

Substrate specificity and kinetic properties of GST-GalT-4 fusion protein

Several different glycosphingolipids were tested as potential acceptors for GST-GalT-4 with UDP-galactose as the donor-substrate. The expressed GST-GalT-4 appears to be highly specific for galactose transfer to an acceptor with a terminal *N*-acetylglucosamine residue at the non-reducing end of the glycosphingolipid. The truncated enzyme has similar substrate specificity with respect to terminal GlcNAc in GSLs and GPs as the native one [62].

The kinetic properties (apparent K_m and observed V_{max} values) of the GST-GalT-4 with respect to UDP-galactose, $MnCl_2$ and the acceptor substrates, Lc3, and pNP-GlcNAc, are listed in Table 1. The apparent K_m values of GST-GalT-4 are comparable to those of purified native GalT-4 from the 11-day-old ECB [24] whereas the V_{max} values of GST-GalT-4 were observed to be 15- to 20-fold lower than those of native ECB GalT-4.

The catalytic properties of GST-GalT-4 and its binding to α-lactalbumin column

The result presented in Table 1 shows that the kinetic parameters of the GST-GalT-4 fusion protein. On binding to α-lactalbumin, GST-GalT-4 lost the ability to transfer galactose from UDP-Gal to pNP-GlcNAc; instead it behaved like lactose synthetase, i.e., it catalyzed the biosynthesis of lactose by transferring galactose from UDP-Gal to glucose. The interaction between the GST-GalT-4 and α-lactalbumin was further confirmed by two methods: by column chromatography of the bacterial cell lysate expressing the GST-GalT-4 fusion protein or the affinity-purified fusion protein through α-lactalbumin coupled to Sepharose (Fig. 5A and 5B) and by a novel solid-phase binding assay as described in the Materials and Methods. An irrelevant GST-fusion protein did not bind to the α-lactalbumin-Sepharose. GST-GalT-4 binds to the α-lactalbumin in the presence of GlcNAc and Mn^{2+}, and the bound fusion protein was eluted from the column with elution buffer containing 20 mM EDTA but lacking GlcNAc and Mn^{2+}, (Fig. 5A). The protein in the eluent was identified as GST-GalT-4 by SDS/PAGE and Western blotting analysis (Fig. 5C). The eluent fraction also exhibited GST (Fig. 5B) and GalT-4 activities ([47]; will be published elsewhere).

In order to facilitate further investigation on the interaction of the recombinant GalT-4 with α-lactalbumin a solid-phase binding assay was developed, as described in the Materials and Methods section. The data obtained from this binding assay (Fig. 6) shows specific interaction of the GST-GalT-4 fusion protein with α-lactalbumin, while an irrelevant GST-fusion protein [47] did not show any binding with α-lactalbumin. The binding of the GST-GalT-4 with α-lactalbumin was also found to increase in a concentration-dependent manner with respect to both α-lactalbumin and GST-GalT-4 (Fig. 6).

Purification of ECB GalT-3 by affinity matrix (Synsorb-57)

The specific oligosaccharide matrix, GalNAcβ1-4Gal-synsorb, known as 'Synsorb

Table 1. Kinetic parameters for truncated GalT-4 (22 kDa) expressed as GST-GalT-4 fusion protein (48 kDa) in *E. coli*.

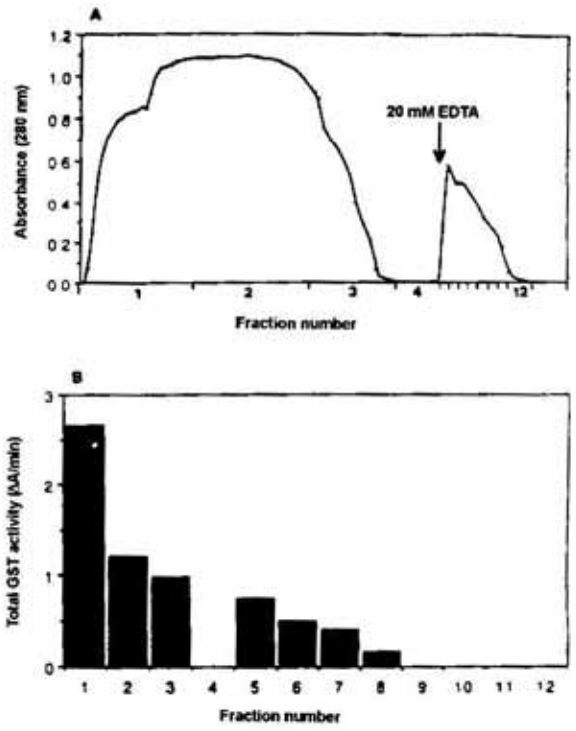
The effect of varying concentrations of the acceptor (LcOse3Cer, SA-Gal-α1-glycoprotein and pNP-GlcNAc) and donor (UDP-[H^3]Gal) substrates on the GalT-4 activity of the fusion protein was measured in the presence of Mn^{2+} (5 mM) using the assay conditions described in Materials and Methods. The range of final concentrations of the acceptor substrates were: LcOse3Cer (0–0.6 mM), pNP-GlcNAc (0–0.5 mM), and SA-Gal-α1-glycoprotein (0–500 μg/ml) while concentration of the donor substrate (UDP-[^{3}H]Gal) was kept constant at 0.2 mM during the determination of apparent K_m and observed V_{max} of the acceptor substrates. In order to determine the apparent K_m and observed V_{max} of UDP-Gal, its concentration was varied up to 0.4 mM; LcOse3Cer (400 μM) was used as acceptor substrate. The effect of Mn^{2+} concentration was measured with LcOse3Cer (400 μM) as an acceptor substrate. The values of apparent K_m and observed V_{max} of each of the substrates were determined from their Lineweaver-Burk plots.

Substrates	K_m (apparent)	V_{max} (observed)
	(μM)	(pmol/h per mg protein)
LcOse3Cer	119	412
SA-Gal-α1 Glycoprotein	172 μg/ml	524
pNP-GlcNAc	122	394
Mn^{2+} (Acceptor LcOse3Cer)	2. 6	418
UDP-Gal (Acceptor LcOse3Cer)	126	398

57' (Chembiomed) was found to be effective in retaining GalT-3 activity. The GalT-3 active fraction, Q-Sepharose eluent fraction (Fig. 7), was dialyzed against buffer A and was allowed to interact with Synsorb 57 beads (pre-equilibrated with the same buffer) at 4°C for at least 4 h with mild agitation. The mixture was applied onto a column and the effluent was collected. The matrix was then washed with the same buffer to remove nonspecifically adsorbed proteins. Then the GalT-3 polypeptide was eluted with a salt gradient from 0 to 0.6 M NaCl, as shown in Fig. 8. The majority of GalT-3 activity was eluted between 0.25 and 0.4 M NaCl. The GalT-3 active eluent fraction were resolved on a preparative 10% SDS/PAGE gel. The GalT-3 polypeptide bands were eluted as described in Materials and Methods after Western blotting based on GalT-3 antibody recognition. The eluent bands were concentrated and lyophilized. The peptides were again SDS/PAGE electrophoresed and electroblotted onto a Immobilon-P (PVDF) solid support in transfer buffer (10 mM Caps, 10% methanol), at 100 volts for 1 h at 4°C. After the transfer onto PVDF solid support, a portion of the blot was immuno-stained and the other part was Coomassie stained. Subsequently, the immuno-stained bands and the Coomassie stained peptides were aligned. Our present data (GalT-3 doublet bands, 62 and 65 kDa) verifies the previously reported values obtained for GalT-3 from ECB [23], as evidenced by SLS (sodium lauryl sarcosinate)/PAGE and activity gel (afer removal of SLS the activity of GalT-3 was determined with 0.2 mm thick sliced gels [23, 24]).

Protein fingerprinting of 19-day ECB peptides with TPCK-trypsin protease

The two protein bands (65 and 62 kDa) that were gel purified, based on anti-GalT-3 recognition, were separated by SDS/PAGE. Each peptide band was subjected to limited proteolysis with TPCK-trypsin, as described under



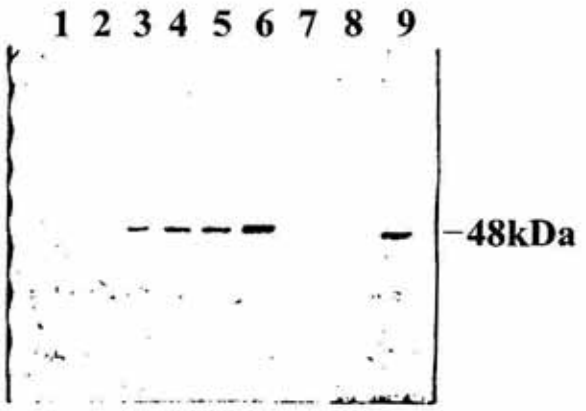


Figure 5. α-Lactalbumin-Sepharose column chromatography of bacterial cell lysate expressing GST-GalT-4 fusion protein.

The profiles (panel A) for elution of protein (absorbance at 280 nm) during chromatography of bacterial cell lysate expressing GST-GalT-4 fusion protein through α-lactalbumin-Sepharose are shown. Chromatographic conditions are described in Materials and Methods. GST activity in the column fractions is shown in panel B. Eluents of the chromatographic were analyzed by Western blot analysis (panel C) with anti-GalT-4 (ECB). Lane 1, fraction 10; lane 2, fraction 9; lane 3, fraction 8; lane 4, fraction 7; lane 5, fraction 6; lane 6, fraction 5; lane 7, fraction 4; lane 8, fraction 3; and lane 9, fraction 1.

Materials and Methods. The digested peptides were resolved on another SDS/PAGE and stained with silver nitrate as shown in (Fig. 9). Both the upper and lower bands gave rise to at least three peptides with identical mobilities, resulting in a similar peptide banding pattern. This suggest a strong amino-acid sequence identity between the two protein bands of ECB GalT-3. Hence, it is highly possible that the lower (62 kDa) peptide is simply a proteolytic cleavage product of the higher (65 kDa) peptide.

GalT-3 is a developmentally regulated enzyme [23, 24] in the embryonic chicken brain. The specific activity of GalT-3 has been shown to increase steadily and to peak at 19 days of age. This pattern of developmental expression for GalT-3 is not at all unusual; it is simply one of several enzymes temporally regulated within the brain. This temporal regulation of the glycosyltransferases functions to control the intricate pattern of glycosphingolipid distribution in the embryonic chicken brain. An example of this highly orchestrated GSL distribution is the polysialogangliosides which have been known to dominate the ealier part of brain development whereas the monosialogangliosides such as G_{M2} and G_{M1} can be significantly detected only in embryos 17 days or older. This explains the rise in the total as well as specific activity of GalT-3 in 19-day-old embryonic chicken brain.

The developmental profile of gangliosides has been widely studied in the embryonic chicken brain [63, 64], obviously as a result of their characteristic tissue-specific expression and their involvement with neuronal function. Therefore, the embryonic chicken brain was used as the model system for biochemical and molecular biological studies of the β1,3-galactosyltransferase (will be published elsewhere). The embryonic chicken brain serves as a suitable animal model for studies related

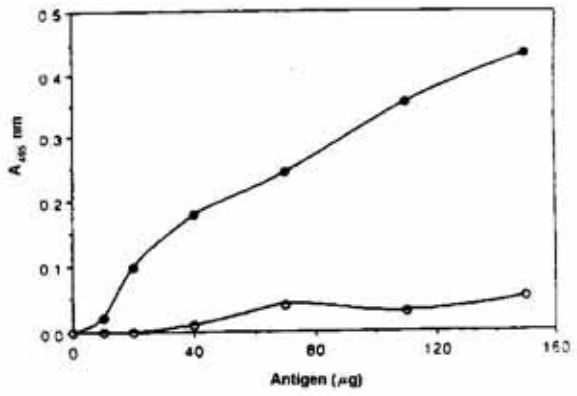


Figure 6. Binding of α-lactalbumin to GST-GalT-4 fusion protein.

The effect of α-lactalbumin concentration on the interaction between α-lactalbumin and GST-GalT-4 fusion protein was determined by the solid-phase binding assay. The assay was done by coating the well with 40 μg of α-lactalbumin, and the absorbance was measured after 30 min incubation of the color reaction. GST-GalT-4 (●), GST-raf (○).

to the developmental stages of an animal brain. Compared to other 'developmental' systems, such as pig and rat embryos, the embryonic chicken brain offers distinct advantages. First, it can be obtained with relative ease and little expense. Second, the procedure for the maintenance and collection of the embryos are extremely simple for the chicken. Third, and may be more important, the embryonic chicken offers the unique opportunity of allowing one to make an evolutionary comparison of a protein or a gene of interest with mammalian systems. However, the embryonic chicken brain is, at best, an approximate model for mammalian systems, and thus careful experimental studies should be carried out before extrapolating information obtained from such a system onto the mammalian species.

GalT-3 was discovered initially in 1965 [48] from embryonic chicken brain. Subsequently, the enzyme activity was detected in neuronal and hepatic tissues from other species [49–52]. However, further studies regarding the purification and characterization of this crucial ganglioside biosynthetic enzyme have been stalled directly as a consequence of the unavailability of sufficient quantities of the acceptor substrate, G_{M2}. Unlike G_{M1} it is not a widely occuring glycosphingolipid. We previously reported an inexpensive and easily available source of β1-3 galactosidase activity in hard shelled clam, *Mercenaria mercenaria*, which was capable of cleaving G_{M1} and yielding intact G_{M2} [65]. Thus, studies on the purification and characterization of GalT-3 were initiated when the sufficient quantities of G_{M2} were accumulated.

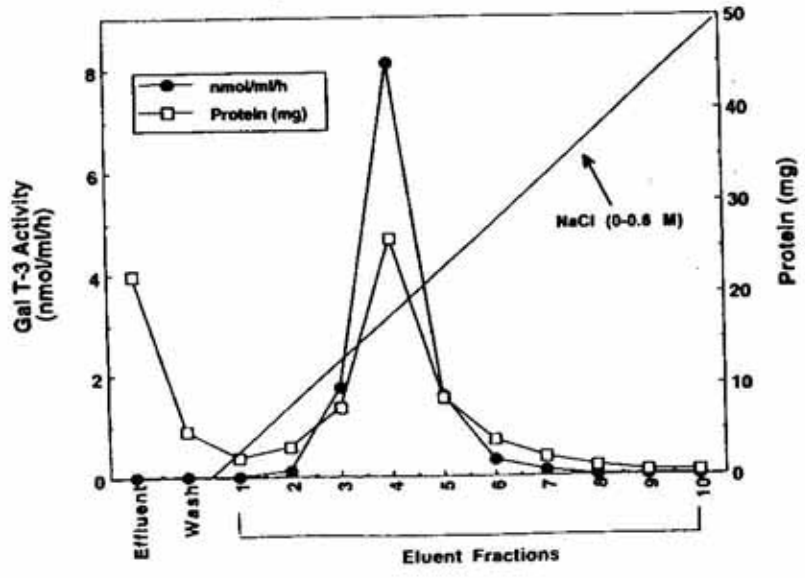


Figure 7. Column chromatography of GalT-3 activity on Q-Sepharose ion-exchange matrix.

A detergent-solubilized extract of 19-day ECB containing GalT-3 activity was loaded onto a Q-Sepharose column and chromatographed as described in Methods. The eluent column fractions were assayed for GalT-3 activity as described in the text and Methods. (●), GalT-3 activity, and (□), protein.

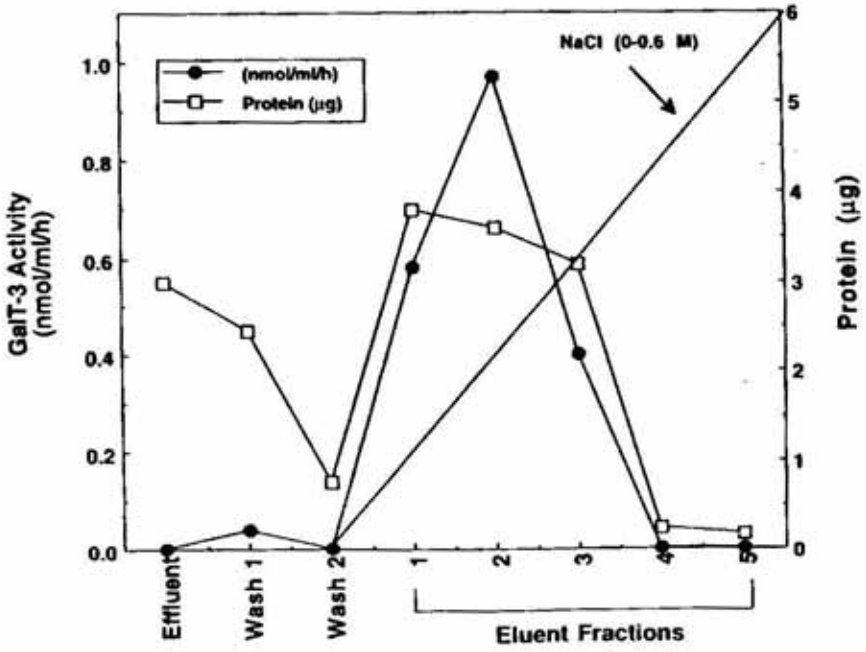


Figure 8. Column chromatography of GalT-3 activity on Synsorb GalNAcβ1-4Gal affinity matrix.

A Q-Sepharose column eluent fraction containing GalT-3 activity was used as enzyme source for binding onto the Synsorb GalNAcβ1-4Gal affinity beads. Chromatographic conditions were as described in the text. GalT-3 activity was assayd in all the fractions as described in the Methods. (●), GalT-3 activity, (□), protein.

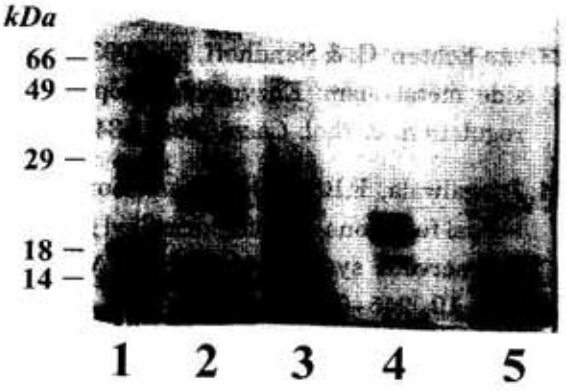


Figure 9. Protein fingerprinting of GalT-3 peptides after treatment with TPCK-trypsin.

Experimental conditions for electrophoresis and protease digestion have been described in Methods. Approximately 0.5 μg of each of the two GalT-3 peptides was digested with TPCK-trypsin. Lane 1, Silver stain standards (BSA, 66 kDa; fumarase, 48. 5 kDa; carbonic anhydrase, 29 kDa; β-lactoglubulin, 18.4 kDa; α-lactalbumin, 14.2 kDa). Lanes 2, and 3, GalT-3 lower band (62 kDa) after digestion with 0.1 and 0.2 μg of TPCK- trypsin, respectively. Lane 4, TPCK-trypsin 0.3 μg; Lane 5, GalT-3 upper band (65 kDa) after digestion with 0.2 mg of TPCK-trypsin.

Stability studies on GalT-3 were initially conducted to gain information on GalT-3's general behavior to facilitate its purification. GalT-3 was found to be extremely temperature-sensitive, and was to be kept at 4°C for all steps during its purification to maintain its optimal activity.

Purified GalT-3 was treated with PNGase-F, and the deglycosylated peptides were detected by a Western blot, showing the polyclonal anti-GalT-3 was directed against the peptide backbone of the protein and not a carbohydrate moiety. This polyclonal antibody appears to be suitable reagent to be used for further immunoscreening of GalT-3 work with ECB βZAP expression library (will be published elsewhere). Expression of identified cDNAs from ECB for GalT-4 [24, 44, 45, 47] and GalT-3 [66, 67] in an eucaryotic system is essential to obtain the structure function relation of these two enzymes. This information will greatly enhance the research in inherited disease therapy. Further research on expression cloning of a cDNA for the β1,3-galactosyltransferase gene was performed in mouse melanoma B16 cells where G_{A1} (asialo G_{M1}), G_{M1} and G_{D1b} were detected on the cell surfaces after transfection with the cDNA [68].

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