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Purification and characterization of GDP-L-Fuc: *N*-acetyl β-D-glucosaminide α1→6fucosyltransferase from human blood platelets

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Dedicated to Roger W. Jeanloz on the occasion of his 80th birthday.

α-6-L-Fucosyltransferase (α1,6FucT; EC 2.4.1.68) from human platelets, the enzyme that is released into serum during coagulation of blood, was purified 100,000-fold. The purification required three sequential chromatographic steps: chromatofocusing, affinity column chromatography on GnGn-Gp(asialo-aglacto-transferrin glycopeptide)-CH-Sepharose, and gel filtration of Sephadex G-200. The final preparation contained a protein that migrated as a single discrete band M_r of 58,000 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions, and as a single enzymatically active peak M_r of 58,000 in gel filtration. Although the purified enzyme utilized the biantennary GnGn-Gp as substrate, it was twice as active with the triantennary oligosaccharide when the Man α1,3 antenna was substituted with GlcNacβ1,4. On the other hand the tetraantennary oligosaccharide was not a preferred substrate. The K_m values for the substrate asialo-agalactotransferrin-glycopeptide, and GDP-L-fucose were 29 and 28 μM, respectively. The optimum pH of the enzyme was 6.0. The activity of α1,6FucT was abolished in the presence of β-mercaptoethanol. Divalent cations such as Mg^{2+} and Ca^{2+} activated, but Cu^{2+}, Zn^{2+} and Ni^{2+} strongly inhibited the activity.

***Keywords*: α1,6-fucosyltransferase, platelets**

Introduction

α1,6FucT (GDP-L-Fuc:*N*-acetyl-β-D-glucosaminide α-6-fucosyltransferase; EC 2.4.1.68) transfers fucose to position 6 of the asparagine-linked *N*-acetylglucosamine residue of N-glycans. In human serum, 95% of the activity of α1,6FucT is derived from blood platelets [1]. Platelets release the enzyme during coagulation of blood or after treatment with agonists that cause a shape change and secretion [2]. The biological significance of α1,6FucT release from platelets is unknown, but in platelets fucosyl residues in α1,6 linkage to the core GlcNAc are present in glycans of the GpIIb-IIIa complex [3] that is essential for fibrinogen binding.

Superficially, liberation of α1,6FucT from platelets by different agonists resembles the release reaction in which low- and high-molecular weight materials contained in a soluble form in platelet granules are secreted. This similarity may be misleading since glycosyltransferases are present in a soluble form in blood serum and other body fluids but it is known that these enzymes are located primarily within cells and are membrane-bound. Solubilization occurs by proteolytic cleavage between the membrane-binding and catalytic domains [4]. The aggregation of platelets is accompained by the activation of endogenous proteases (calpains) [5]. Thus, the release of α1,6FucT from platelets may result either from secretion of a soluble enzyme contained within granules or proteolysis of a membrane-bound form or both [2].

The enzyme-active platelets appear to represent only some subpopulations of platelets. It is likely that these platelets contain Golgi since according to observations by electron microscopy, the complex is present only in some platelets [6]. Kościelak *et al.* [7] suggested that α1,6FucT of platelets may be a marker of the ploidy level of megakaryocytes because the enzyme activity is highly increased in platelets under conditions of thrombocytopenia.

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Thus, the enzyme activity in platelets may have some diagnostic value.

Schachter *et al.* [8, 9] first described α1,6FucT from porcine liver using a partially purified enzyme extract. The α1,6FucT from cultured human skin fibroblasts of a cystic fibrosis patient was subsequently purified to electrophoretic homogeneity and characterized by Voynow *et al.* [10]. Two proteins, M_r 39 000 and 34 000, were seen in SDS-polyacrylamide gel electrophoresis. Recently, Uozumi *et al.* [11] have reported the purification, characterization and cloning of α1,6FucT from porcine brain. The brain α1,6FucT had 575 amino acids and no putative N-glycosylation sites. The enzyme was highly expressed in rat brain. The α1,6FucT from porcine brain may be a different enzyme from the human fibroblast α1,6FucT, because it has a different pH optimum and size [11]. Most recently, α1,6FucT was purified, characterized and cloned from a culture supernatant of a human gastric cancer cell line, MKN45 [12]. The homology to porcine brain α1,6FucT was 92.2%. No sequence homology was found with previously cloned α1,2, α1,3, and α1,4fucosyltransferases.

In a view of a possible clinical relevance of α1,6FucT activity in platelets and different properties of the enzymes from various sources, in the present study we purified and characterized α1,6FucT from human platelets.

Materials and methods

Chemicals and reagents

GDP-L-[^{14}C] fucose was obtained from Amersham. (Buckinghamshire, UK). PBE 94, polybuffer 96, CH-activated Sepharose 4B, Sephadex G-200 were purchased from Pharmacia (Uppsala, Sweden). Ethanolamine, Triton CF 54, human apo-transferrin, glycerol, EDTA, molecular weight standards, phenylmethylsulfonyl fluoride (PMSF), and GMP were from Sigma (Deisenhofen, Germany). Acrylamide, N,N′-bisacrylamide were obtained from Merck (Darmstadt, Germany). Trimannosyl core, asialo-agalacto-biantennary, asialo-agalacto-triantennary, asialo-agalacto-tetraantennary were from Oxford Glyco-Systems (Abingdon, UK). Asialo-agalactotransferrin-glycopeptide (GnGn-Gp) was prepared as described [13] and used either as a substrate or an affinity ligand to CH-activated Sepharose 4B [10] for affinity purification of the enzyme. GDP-hexanolamine-Sepharose was a kind gift of Dr. M. Blaszczyk. Other chemicals were of the finest grade available. BCS-Na Biodegradable Counting Scintillant (Amersham) was used for radioactive counting.

Preparation of platelets

Blood withdrawn from regular blood donors was collected into 1/10 volume of 3.8% sodium citrate. Platelets were isolated by the method described [14] and washed with 10 mM Tris, pH 7.4 containing 150 mM NaCl. Subsequently, platelets were suspended in 50 mM cacodylate buffer, pH 6.0 with 40 mM $MgCl_2$, 10% glycerol and 0.2 mM PMSF (suspension buffer).

Purification of α-6-L-fucosyltransferase

Platelets from 450 ml of blood were sonicated at 4 °C ten times, each lasting for 10 sec, and then centrifuged at 11 700 × g for 10 min. The supernatant solution was decanted and centrifuged at 105 000 × g for 1 h. The pellet was resuspended in the suspension buffer and then extracted by gentle stirring for 1 h, followed by centrifugation at 105 000 × g for 1 h. The supernatant solution was dialysed against 300 ml of 25 mM ethanolamine, pH 9.4 for 20 h.

Chromatofocusing

The dialyzed preparation was applied to a column of PBE 94 (1.0 cm × 7.0 cm) equilibrated in 25 mM ethanolamine, pH 9.4 with 10% glycerol, and 0.1% Triton CF 54. The PBE column was washed with the 25 mM ethanolamine buffer as above and then eluted with Polybuffer 96, pH 6.0, 10% glycerol, 0.1% Triton CF 54. Fractions of 2 ml were collected and pH measurements were made at 4 °C. The fractions were combined within a specific range, pH 7.7 to 8.0.

Affinity chromatography

The active fractions from the chromatofocusing column were made to 30 μM GMP and applied to the asialo-agalactotransferrin-glycopeptide-CH-Sepharose 4B column (1.2 cm × 1.8 cm). The column was previously equilibrated with 25 mM Tris-HCl buffer, pH 7.5, containing 10% glycerol, 0.1% Triton CF 54 (Buffer A) containing 30 μM GMP at a flow rate 0.5 ml per 1 min. The unbound material was reapplied and the column was washed with Buffer A. The column was then eluted successively with 2 ml and 3 ml portions of Buffer A containing 1 M NaCl. The enzyme-containing fraction (3 ml) which eluted from the affinity column was collected at 5 °C and dialyzed for 20 h against Buffer A containing 5 mM $MgCl_2$.

Sephadex G-200 chromatography

The fraction from the affinity column was concentrated to 0.7 ml using an Amicon YM 30 membrane and then applied to a Sephadex G-200 column (2.0 cm × 40 cm) equilibrated in 50 mM cacodylate buffer, pH 7.0 containing 10 mM $MgCl_2$. 10% glycerol, 0.1% Triton CF 54. The column was eluted with the same buffer and concentrated using an Amicon YM 30 membrane. Standards of chymotrypsinogen A, ovalbumin and bovine serum albumin (BSA) marked the positions of M_r 25,000, 43,000 and 66,000 respectively.

Assay for α1,6FucT

The standard incubation mixture for α1,6FucT assay contained the following components, in the final volume 28 μl:

50 mM cacodylate buffer, pH 6.0 with 10 mM $MgCl_2$, 10 mM EGTA, 61 pmol GDP-[^{14}C]fucose, 5 μg GnGN-Gp [2]. The reaction was started with 6 μl of enzyme extract. The standard conditions of the reactions were: time 1 h, temperature 37 °C. Samples were then cooled to 4 °C, aliquots directly spotted on DE-81 Whatman paper, and separated by descending chromatography with water. The solvent was allowed to run to a distance of 16 cm from the origin. Thereafter, the papers were dried and developed once again with methanol-ethanol (3 : 2) to 26 cm from the origin. Radioactivity present on four 1 × 4 cm adjacent paper strips at 15–19 cm from the origin was determined by liquid scintillation counting.

Preparation of asialo-agalactotransferrin-glycopeptide as substrate and affinity column

GnGn-Gp was prepared from apotransferrin as described previously [13] and used either as substrate or coupled to CH-Sepharose 4B for use as an affinity column [10].

Polyacrylamide gel electrophoresis

Protein was precipitated with acetone at −20 °C, and resuspended in electrophoretic sample buffer. SDS-PAGE was performed according to Laemmli [15], with the use of 10% acrylamide (Sigma, Deisenhofen, Germany). Staining of the gels with silver nitrate followed the standard protocol of Bio-Rad. Standards were carbonic anhydrase, fumarase, and bovine serum albumin (BSA), M 29,000, 48,500 and 66,000, respectively (Sigma, Deisenhofen, Germany).

Results

Purification of α1,6FucT

α1,6FucT from human platelets was purified using, in part, the procedure described for human skin fibroblasts [10]. A Triton CF-54 extract of the microsomal preparation of platelets, which contained only membrane-bound enzyme, showed multiple protein bands (Figure 1, lane A). The Triton CF-54 extract was then subjected to chromatofocusing on the PBE94 column. α1,6FucT was eluted from the column with range of pH 7.7 to 8.0. The optimal range of 7.9 indicated a slightly basic pl for the enzyme. The enzymatically active protein was further purified with the use of an affinity column, GnGn-Gp-Sepharose 4B [10] (see Materials and methods). A comparison of the protein composition of the purified fraction by SDS-PAGE (Figure 1, lane C) with that of the fraction obtained after chromatofocusing (Figure 1, lane B) showed the presence of only four major proteins M_r 98, 84, 58, and 39 kDa.

Final purification of α1,6FucT was accomplished by filtration on Sephadex G-200 (Figure 2) which yielded a single enzyme active fraction with a specific activity of 455×10^{-3} μmoles Fuc min^{-1} mg $protein^{-1}$, and M_r 58 kDa. Moreover, the purification of the α1,6FucT was 100,000-fold

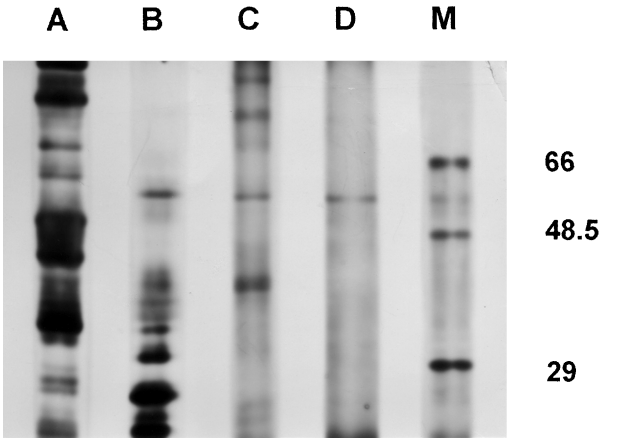


Figure 1. SDS-PAGE profile of the purified α-6-L-fucosyltransferase from human platelets. Electrophoresis was performed in 10% polyacrylamide gel under non-reducing conditions. Fractions obtained at different stages of the purification procedure were stained with silver nitrate. (A), Triton CF 54 extract of the microsomal preparation; (B), fraction obtained by chromatofocusing, pH 7.7–8.1; (C), fraction eluted from the affinity column; (D), active fraction from Sephadex G-200 column. (M), Marker proteins were: bovine serum albumin (66 kDa) fumarase (48.5 kDa), carbonic anhydrase (29 kDa).

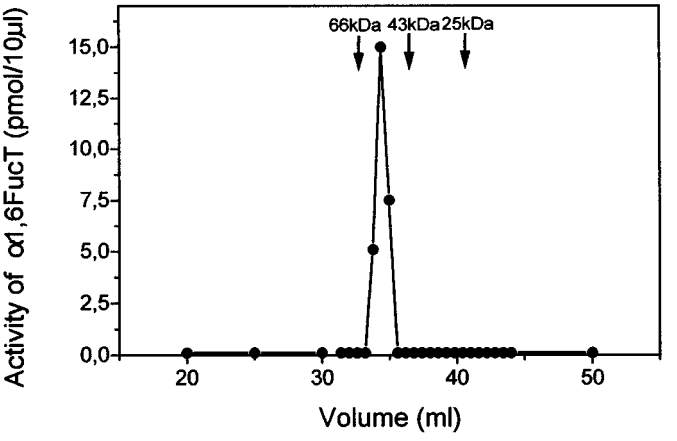


Figure 2. Gel filtration on Sephadex G-200 of affinity purified α-6-L-fucosyltransferase. Arrows indicate positions of molecular weight standards (66 kDa, bovine albumin; 43 kDa, ovalbumin; 25 kDa, chymotrypsinogen). Void volume of the column was 20 ml.

in approximately 7% yield from the starting platelet material. SDS-PAGE (Figure 1, lane D) of the purified fraction stained with silver nitrate showed a single discrete protein band of M_r 58 kDa. In the light of the gel filtration experiment this band represented enzymatically active protein. The enzyme did not bind to GDP-hexanolamine-Sepharose (data not shown). Details of the purification steps, specific activity, recovery and degree of purification of the α1,6FucT are summarized in Table 1.

Table 1. Purification of *α*-6-L-fucosyltransferase from human platelets.

Step	*Protein (mg)*	*Total Activity (nmol Fuc h^{-1})*	*Specific Activity [nmol Fuc min^{-1} protein^{-1}]*	*Purification*	
				-fold	*Yield (%)*
Platelets	60.0	15.60	0.0043	1	100
Triton CF-54 extract	2.8	9.80	0.058	13.5	53
Chromatofocusing	0.3	4.68	0.26	60	30
GnGn-Gp-Sepharose	0.02	2.80	2.33	538	17
Sephadex G-200	0.00004	1.07	455	102,690	6.8

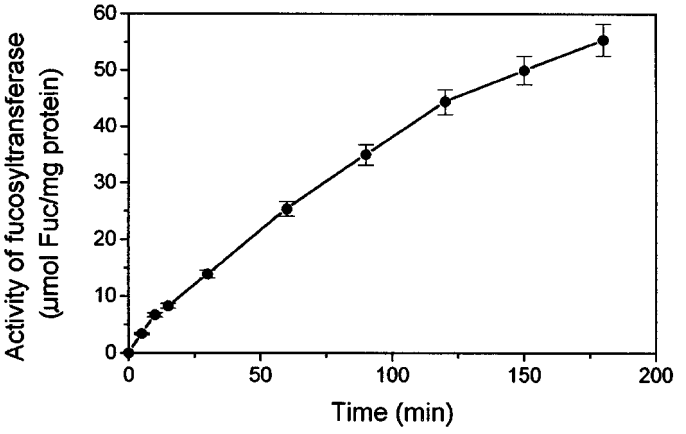


Figure 3. Enzymatic transfer of [^{14}C]fucose from GDP-[^{14}C]fucose to asialo-agalactotransferrin-glycopeptide by *α*-6-L-fucosyltransferase as a function of time. Data are the means ± S.D. of three independent experiments.

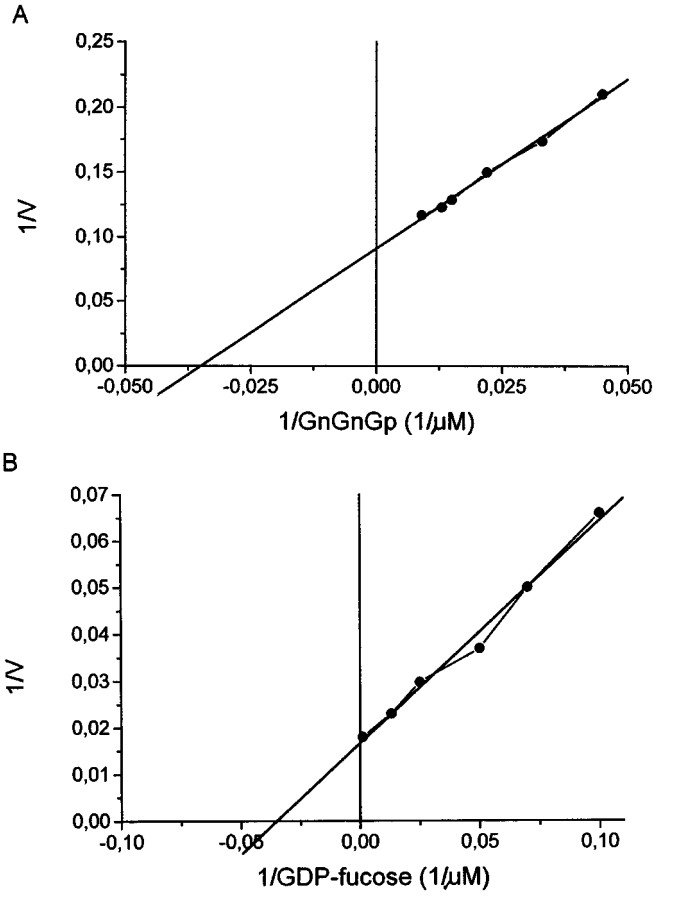


Figure 4. Lineweaver-Burk plot of *α*-6-L-fucosyltransferase activity with varying concentrations of (A) asialo-agalactotransferrin glycopeptide, and (B) GDP-L-fucose.

Biochemical characterization of α1,6FucT

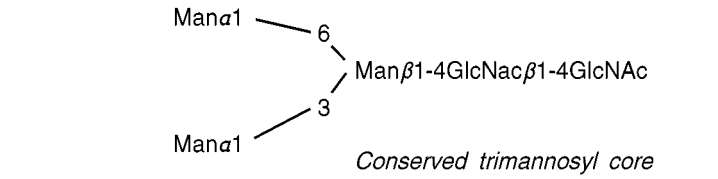
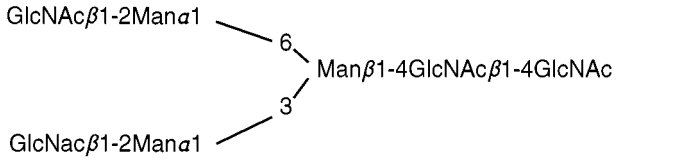
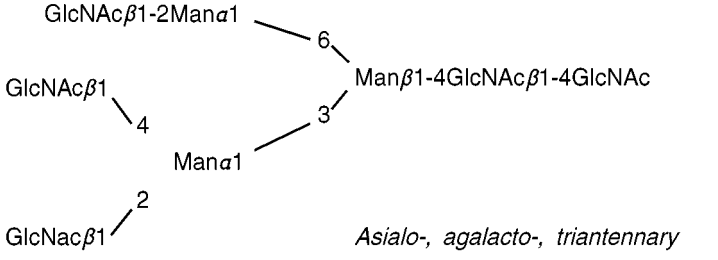
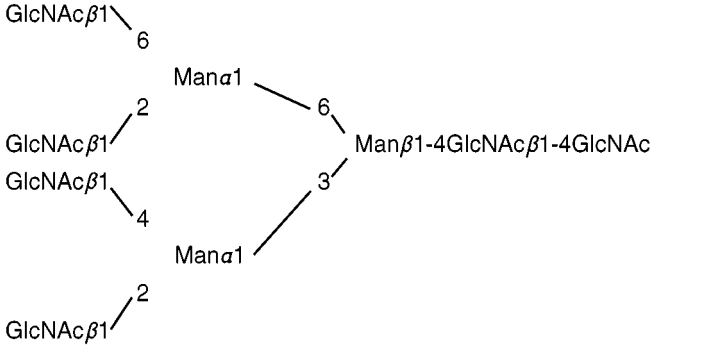
Using GnGn-Gp as substrate to detect the activity of α1,6FucT, the parameters of time and substrate concentration were examined. The rate of [^{14}C]fucose transfer to GnGn-Gp substrate was constant up to at least 2 h at 37 °C (Figure 3). The K_m values for the substrate asialo-agalactotransferrin-glycopeptide, and GDP-L-fucose were 29 μM and 28 μM, respectively (Figure 4).

The requirement of the enzyme for GlcNAc terminated bi, tri, or tetraantennary *N*-glycans as substrates was investigated using commercially available glycans (Table 2). All substrates were studied at 50 μM concentration. The enzyme had the highest activity with the triantennary glycopeptide and two times less activity with the biantennary glycopeptide. The tetraantennary glycopeptide was considerably less active. The activity of asialo-agalactotransferrin-glycopeptide and that of the corresponding oligosaccharide were similar within the range of experimental error.

The optimum pH for the activity of the platelet α1,6FucT was 6.0 (Figure 5) and was broad (pH 4.5 to 8.5). The optimal buffers for purified enzyme were cacodylate and Mes when compared to Tris-HCl and sodium phosphate buffers, pH 7.0.

The effect of divalent cations upon the enzyme activity is shown in Figure 6. All cations were studied at a fixed concentration (10 mM). α1,6FucT was active in the absence of divalent metal ions, but was stimulated upon addition of Mg^{2+} and Ca^{2+}, and strongly inhibited in the presence of Ni^{2+}, Cu^{2+} or Zn^{2+}. Mn^{2+} was found to have negligible

Table 2. Substrate specificty of α1,6FucT from human platelets.

Structure of substrate	*Activity of α1,6Fuct (nmol h^{-1} mg protein^{-1})*
 *Conserved trimannosyl core*	No activity
 *Asialo-, agalacto-, biantennary*	29 000
 *Asialo-, agalacto-, triantennary*	59 450
 *Asialo-, agalacto-, tetraantennary*	2 400

effect on the activity of the enzyme. The enzyme was active in the presence of EDTA.

The influence of β-mercaptoethanol on the activity of the enzyme was investigated. The activity of enzyme was reduced by approximately 20, 70 and 95% in the presence of 2, 5 and 10 mM β-mercaptoethanol, respectively.

Discussion

α1,6FucT was purified from human platelets to electrophoretic homogeneity as a protein, M_r 58 kDa. The optimum pH 6.0, and Km values of 28 μM and 29 μM for GDP-L-fucose and asialo-agalactotransferrin-glycopeptide, respectively, were determined. The enzyme activity was inhibited by Ni^{2+}, Cu^{2+}, and Zn^{2+} cations. Thus, α1,6FucT from platelets was quite similar to α1,6FucT from porcine brain and human gastric cancer that were also inhibited by the same cations [11, 12]. There were, however, differences in that the platelet enzyme was activated (50%) by Ca^{2+} and Mg^{2+}, unlike porcine brain and gastric cancer enzymes. Moreover, the platelet enzyme like the fibroblast enzyme [10] was not bound to GDP-hexanolamine-Sepharose. The

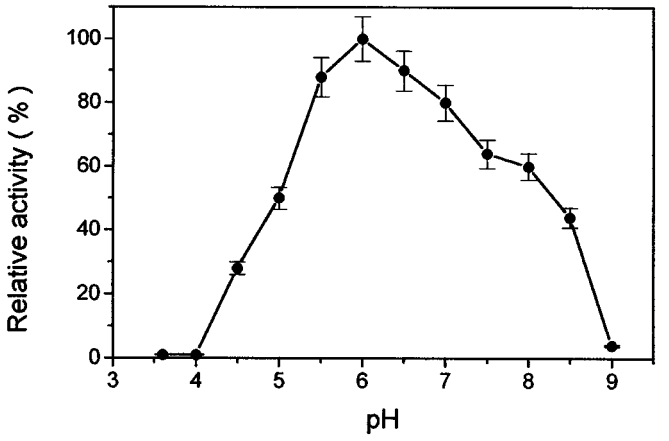


Figure 5. Effect of pH on the activity of *α*-6-L-fucosyltransferase. The pH values were obtained with 100 mM acetate/NaOH (pH 3.6–5.5), Mes/NaOH (5.5–7.0), Tris-HCl (7.0–9.0) buffers. Data are means ± S.D. of three independent experiments.

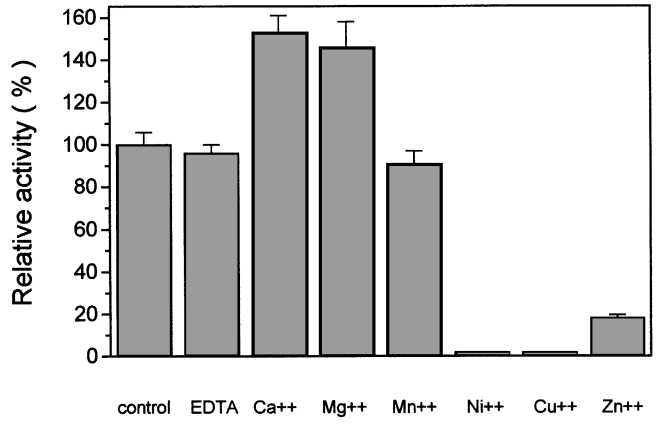


Figure 6. Effect of divalent cations and EDTA on the activity of *α*-6-L-fucosyltransferase of human platelets. EDTA and cations were used at 10 mM concentrations. Data are means ± S.D. of three independent experiments.

enzyme from human fibroblasts migrated in SDS-PAGE under denaturing conditions as a doublet, M_r 34 and 39 kDa [10]. It remains to be established if the enzymes from the different sources represent a family of α1,6FucTs.

The substrate specificity of the platelet enzyme was quite similar to α1,6FucT from porcine liver [9]. The peptide portion of N-linked glycoproteins was not required for enzyme activity as previously reported [10]. Interesting however, was that the addition of one antenna to a Manα-6 arm of a triantennary oligosaccharide almost completely abolished its ability to serve as substrate for the enzyme. Recently, a nodZ gene present in soil bacteria has been shown to be an α1,6FucT utilizing chitin oligosaccharides as substrate [16]. It should be noted that chitin affinity chromatography was not useful to purify the fibroblast α1,6FucT or as a substrate for the fibroblast enzyme [10].

It will be necessary to examine the sequence homology of α1,6FucT from different sources in order to determine whether or not a family of enzymes exist and to obtain a better understanding of the evolution and functions of these enzymes.

Acknowledgments

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