J. Biochem. 2010;147(6):851–856 doi:10.1093/jb/mvq019

JB THE JOURNAL OF BIOCHEMISTRY

Discrimination of porcine glycogen debranching enzyme isozymes by the ratios of their 4-α-glucanotransferase and amylo-α-1,6-glucosidase activities

Received February 3, 2010; accepted February 10, 2010; published online February 17, 2010

Seiko Doi, Yasushi Makino and Kaoru Omichi*

Department of Chemistry, Graduate School of Science, Osaka Prefecture University, 1-1, Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan

*Kaoru Omichi, Department of Chemistry, Graduate School of Science, Osaka Prefecture University, 1-1, Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan. Tel: +81 72 254 9191, email: komichi@c.s.osakafu-u.ac.jp

Glycogen debranching enzyme (GDE) is a single-chain protein containing distinct active sites that exhibit 4-α-glucanotransferase and amylo-α-1,6-glucosidase activities. The ratios of these two activities in porcine liver and muscle GDEs were compared using a set of homologous fluorogenic branched dextrins. For quantifying 4-α-glucanotransferase activity, 6^3-*O*-α-maltotetraosyl-PA-maltooctaose (B3/84), 6^4-*O*-α-maltotetraosyl-PA-maltooctaose (B4/84), 6^5-*O*-α-maltotetraosyl-PA-maltooctaose (B5/84) and 6^6-*O*-α-maltotetraosyl-PA-maltooctaose (B6/84) were used as substrates and maltohexaose (G6) as the acceptor. The substrate for amylo-α-1,6-glucosidase activity was 6^3-*O*-α-glucosyl-PA-maltotetraose (B3/41). HPLC analysis of the fluorogenic branched dextrin digests in the presence of G6 revealed that GDE 4-α-glucanotransferases produce the corresponding 6-*O*-α-glucosyl-PA-maltooctaose (GG8PA) and maltononaose (G9). The ratios of the 4-α-glucanotransferase activity to amylo-α-1,6-glucosidase activity, for the liver and muscle enzymes were respectively 0.240 and 0.0840 for B3/84, 0.204 and 0.0788 for B4/84, 0.145 and 0.0592 for B5/84, and 0.109 and 0.0458 for B6/84. These data clearly indicate that porcine liver and muscle GDEs are different from each other. The ratios of porcine brain GDE were 0.155, 0.131, 0.0990 and 0.0745 for B3/84, B4/84, B5/84 and B6/84, respectively. These results indicate that porcine brain GDE is also unique from liver and muscle enzymes, suggesting that it is either a third enzyme, or a mixture of 45% liver and 55% muscle GDEs.

Keywords: brain/branched dextrin/glycogen/glycogen debranching enzyme/transglycosylation.

Abbreviations: B3/41, 6^3-*O*-α-glucosyl-PA-maltotetraose; B3/81, 6^3-*O*-α-glucosyl-PA-maltooctaose; B3/84, 6^3-*O*-α-maltotetraosyl-PA-maltooctaose; B4/81, 6^4-*O*-α-glucosyl-PA-maltooctaose; B4/84, 6^4-*O*-α-maltotetraosyl-PA-maltooctaose; B5/81, 6^5-*O*-α-glucosyl-PA-maltooctaose; B5/84, 6^5-*O*-α-maltotetraosyl-PA-maltooctaose; B6/81, 6^6-*O*-α-glucosyl-PA-maltooctaose; B6/84, 6^6-*O*-α-maltotetraosyl-PA-maltooctaose; EDTA, ethylenediaminetetraacetate; GDE, glycogen debranching enzyme; Glc, D-glucose; GlcPA, 1-deoxy-1-[(2-pyridyl)amino]-D-glucitol; G4PA, PA-maltotetraose; G6, maltohexaose; G9, maltononaose; G8PA, PA-maltooctaose; GG8PA, 6-*O*-α-glucosyl-PA-maltooctaose; PA, pyridylamino.

Glycogen debranching enzyme (GDE) is found in the liver and muscle, and degrades glycogen in concert with glycogen phosphorylase (*1*, *2*). The enzyme has distinct active sites for 4-α-glucanotransferase (1,4-α-glucan:1,4-α-glucan 4-α-glycosyltransferase, EC 2.4.1.25) and amylo-α-1,6-glucosidase (dextrin 6-α-glucosidase, EC 3.2.1.33) activities on the single polypeptide chain (*3*–*9*).

The human genome contains one GDE gene and at least two promoter regions. In liver and muscle, isoform mRNAs are differentially expressed in a tissue-specific manner (*10*). The amino acid sequences of the liver and muscle forms of the enzyme show dissimilarity, indicating differences in the three-dimensional structures of the two active sites.

Glycogen is present in brain as well as in liver and muscle, however, little is known about its degradation. Recently, we purified GDE from porcine brain (*11*), as porcine brain GDE has both 4-α-glucanotransferase and amylo-α-1,6-glucosidase activities, indicating its involvement, together with glycogen phosphorylase, in the degradation of brain glycogen (*12*, *13*). Considering the physiological function of the brain, this brain enzyme is expected to have different substrate specificity compared to liver or muscle form as human liver GDE differs from its muscle counterpart. The biochemical feature of porcine brain GDE including the amino acid sequence and substrate specificity has not been elucidated, and it remains unclear whether brain GDE is the same enzyme as that found in liver or muscle.

Substrates are probes for inspecting the active sites of enzymes, and they are expected to differentiate the active sites of the three GDEs. We previously reported 6-*O*-α-maltotetraosyl-PA-maltooctaoses as substrates for 4-α-glucanotransferase, and 6^3-*O*-α-glucosyl-PA-maltotetraose as a substrate for amylo-α-1,6-glucosidase (*14*, *15*). This set of homologous fluorogenic branched dextrins should be useful for investigating differences in the active sites of the three GDEs because the fluorogenic products can be sensitively

and accurately quantified by HPLC, allowing quantitative analysis of their enzymatic activity.

This article reports the difference between porcine liver and muscle GDEs based on the ratio of the 4-α-glucanotransferase and amylo-α-1,6-glucosidase activities, and also discusses the nature of porcine brain GDE.

Materials and Methods

Materials

G6 and Wakosil-II 5C18 HG column (6 × 150 mm) were purchased from Wako Pure Chemicals (Osaka, Japan).

Porcine liver, muscle and brain GDEs were purified as described previously (*16*, *17*, *11*). B3/84 [Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-4Glcα1-4Glcα1-4Glcα1-6)Glcα1-4Glcα1-4GlcPA], B4/84 [Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-4Glcα1-4Glcα1-4Glcα1-6)Glcα1-4Glcα1-4Glcα1-4GlcPA], B5/84 [Glcα1-4Glcα1-4Glcα1-4(Glcα1-4Glcα1-4Glcα1-4Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA], B6/84, [Glcα1-4Glcα1-4(Glcα1-4Glcα1-4Glcα1-4Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA] and B3/41 [Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4GlcPA] were prepared as described previously (*14*, *15*). The standard compounds for HPLC, B3/81 [Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4GlcPA], B4/81 [Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4GlcPA], B5/81 [Glcα1-4Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA], B6/81 [Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA], PA-maltotetraose (G4PA, Glcα1-4Glcα1-4Glcα1-4GlcPA) and PA-maltooctaose (G8PA, Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA), which were prepared previously, were used (*15*).

Reaction of GDE with fluorogenic branched dextrin

A mixture of 15 μM fluorogenic branched dextrin (B3/84, B4/84, B5/84, or B6/84) and 0.5 mM G6 was incubated at 37°C for an appropriate period with porcine liver, muscle or brain GDE in 40 μl of 50 mM sodium maleate buffer (pH 6.0) containing 0.05% gelatin, 5 mM EDTA and 10 mM β-mercaptoethanol. The concentration of the enzyme was adjusted so that more than 94% of the substrate remained when the reaction was terminated. To stop the enzymatic reaction, 40 μl of 0.1 M acetic acid was added and the mixture was heated at 100°C for 5 min. The enzymatic reaction mixture was analysed by reversed phase HPLC.

Product analysis by HPLC

The fluorogenic products in the enzymatic reaction mixtures were separated and quantified by HPLC using a Wakosil-II 5C18 HG column (6.0 × 150 mm). The elution buffer was 50 mM ammonium acetate, pH 4.5, containing 0.08% 1-butanol at a flow rate of 1.5 ml/min. Elution was monitored by measuring the fluorescence (excitation wavelength, 320 nm; emission wavelength, 400 nm).

Measurement of the rate of GDE 4-α-glucanotransferase action on fluorogenic branched dextrin

The rate of GDE 4-α-glucanotransferase action on fluorogenic branched dextrins was calculated from the total amount of GG8PA and G8PA liberated in the enzymatic reaction mixtures.

Measurement of the rate of GDE amylo-α-1,6-glucosidase action on B3/41

The GDE amylo-α-1,6-glucosidase activity was measured using B3/41 as the substrate. B3/41 (15 μM) was incubated at 37°C for 15 min with porcine liver, muscle or brain GDE in 40 μl of 50 mM sodium maleate buffer (pH 6.0) containing 0.05% gelatin, 5 mM EDTA and 10 mM β-mercaptoethanol. The concentration of each enzyme was adjusted to hydrolyse less than 6% of the B3/41. The enzymatic reaction was stopped by adding 40 μl of 0.1 M acetic acid and then heating the mixture at 100°C for 5 min. G4PA liberated in the reaction mixture was separated and quantified by HPLC as described above. The rate of GDE amylo-α-1,6-glucosidase action on B3/41 was calculated from the amount of G4PA liberated.

Calculation of the ratio of 4-α-glucanotransferase and amylo-α-1,6-glucosidase activities

The ratio of the GDE 4-α-glucanotransferase activity to amylo-α-1,6-glucosidase activity for each of the three GDEs was determined from the rate of 4-α-glucanotransferase action on fluorogenic branched dextrins (B3/84, B4/84, B5/84 or B6/84) relative to the rate of amylo-α-1,6-glucosidase action on B3/41.

Results and discussion

Modes of action of porcine liver and muscle GDEs on fluorogenic branched dextrins

B3/84, B4/84, B5/84 or B6/84 was incubated with porcine liver or muscle GDE for 15 min in the presence of G6. The mixture at an early stage of the enzymatic reaction was analysed by HPLC.

In all reaction mixtures, GG8PA and G8PA were found as the fluorogenic products (Fig. 1). Product analysis by HPLC indicated that GDE 4-α-glucanotransferase removes the maltotriosyl residue from the maltotetraosyl branch, producing the corresponding GG8PA. The maltotriosyl residue is then transferred to the non-reducing-end glucosyl residue of G6 to generate G9. Some GG8PA is subsequently hydrolysed by the GDE amylo-α-1,6-glucosidase to the debranched product (G8PA) and glucose (Fig. 2). These results suggest that the GDE 4-α-glucanotransferase active site strictly recognizes the maltotriosyl residue and the glucosyl residue of the maltotetraosyl branch and the branching-point glucosyl residue of the PA-maltooctaosyl residue.

The mode of action of liver GDE on B4/84 in the presence of maltohexaose was consistent with that previously reported (*18*).

Differences depending on the substrate were observed in the ratio of GG8PA to G8PA liberated. This presumably reflects the rate of 4-α-glucanotransferase action on the dextrins and the rate of amylo-α-1,6-glucosidase action on GG8PA.

Susceptibility of the fluorogenic branched dextrins towards the two GDEs

Since the product (GG8PA) of the 4-α-glucano transferase action on the fluorogenic branched dextrin was hydrolysed to G8PA and glucose by amylo-α-1,6-glucosidase, the rate of 4-α-glucanotransferase action could be calculated from the total amount of GG8PA and G8PA. In order to confirm this estimate, the time course of the liver and muscle GDE actions on the fluorogenic branched dextrins was examined; Fig. 3 shows a representation of the action of liver GDE on B3/84. The total amount of GG8PA (B3/81 in the case of B3/84 as the substrate) and G8PA increased linearly with time, although the amount of GG8PA alone did not, indicating that the rate of 4-α-glucanotransferase action can be measured from linear increments of the total amount of GG8PA and G8PA.

The relative rates of 4-α-glucanotransferase actions on the fluorogenic branched dextrins are shown in Table I. Irrespective of the enzyme, the rates for B3/84 were the fastest, and decreased in the order: B4/84, B5/84 and B6/84. The relative rate is clearly dependent on the enzyme. The Glcα1-4Glcα1-4(Glcα1-4Glcα1-4Glcα1-4Glcα1-6)Glc structure is

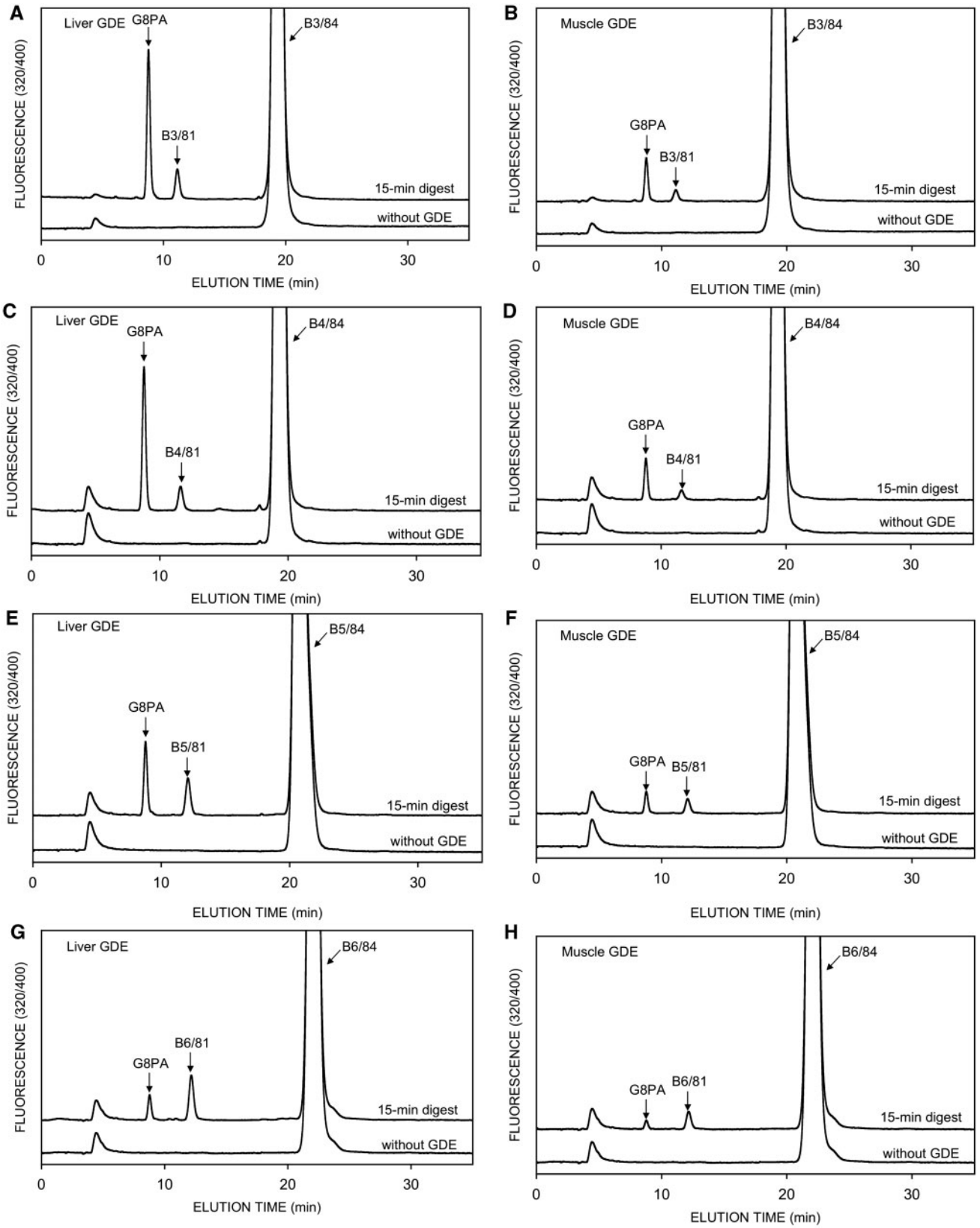

Fig. 1 HPLC of digests of fluorogenic branched dextrins by porcine liver and muscle GDEs. Enzymatic reaction and HPLC of the reaction mixture were carried out as described in 'MATERIALS AND METHODS' section. Chromatograms of reaction mixtures without GDE are shown at the bottom. (A) 15-min digest of B3/84 by liver GDE; (B) 15-min digest of B3/84 by muscle GDE; (C) 15-min digest of B4/84 by liver GDE; (D) 15-min digest of B4/84 by muscle GDE; (E) 15-min digest of B5/84 by liver GDE; (F) 15-min digest of B5/84 by muscle GDE; (G) 15-min digest of B6/84 by liver GDE; (H) 15-min digest of B6/84 by muscle GDE.

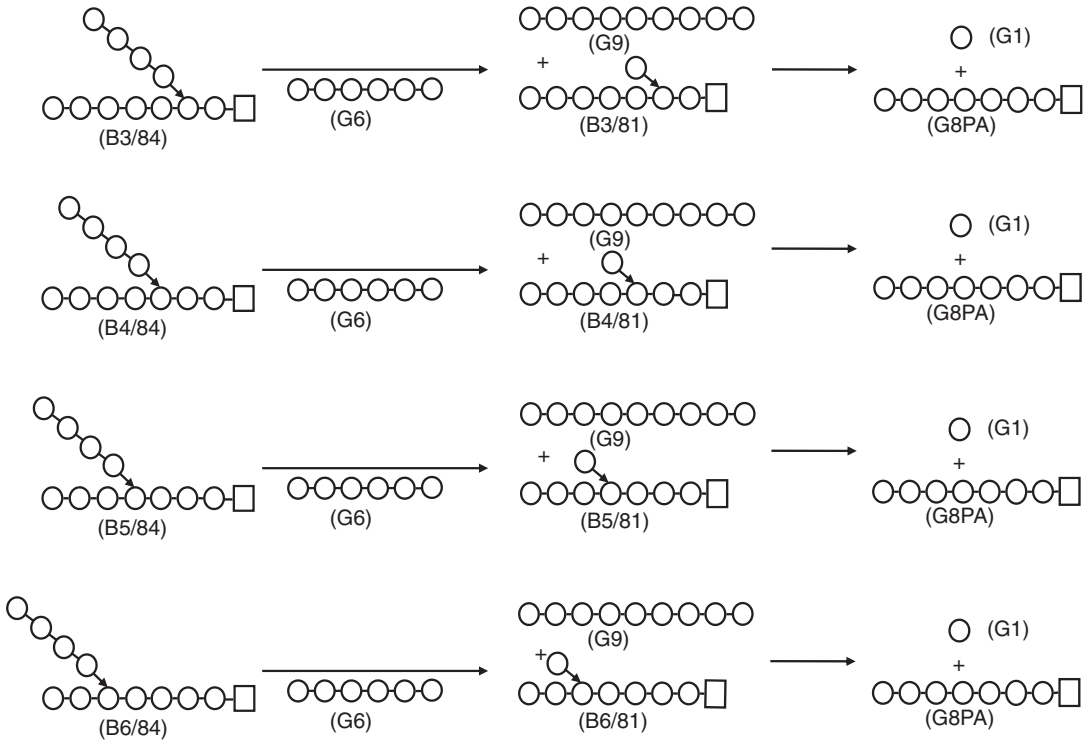

Fig. 2 Mode of action of porcine liver and muscle GDE on fluorogenic branched dextrins. Open circle, D-glucose residue; dash, α-1,4-glycosidic linkage; open square, 1-deoxy-1-[(2-pyridyl)amino]-D-glucitol residue; down arrow, α-1,6-glycosidic linkage.

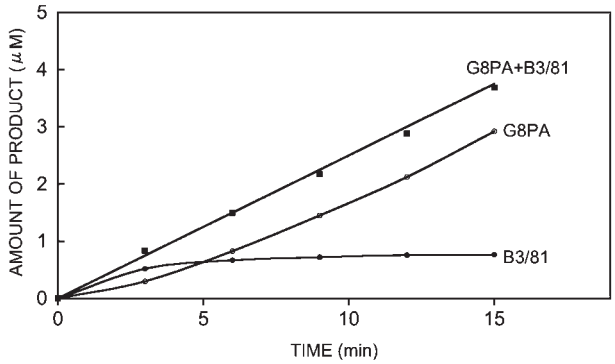

Fig. 3 Time course of porcine liver GDE action on B3/84. B3/84 was digested with liver GDE in the presence of G6, and the reaction mixture was analysed by HPLC. Open circle, G8PA; filled circle, B3/81; filled square, total of G8PA and B3/81.

Table I. Relative rates of the 4-α-glucanotransferase actions of liver and muscle GDEs on fluorogenic branched dextrins.

Substrate	Relative rate	
	Liver GDE	Muscle GDE
B3/84	1.00[a]	1.00[a]
B4/84	0.85	0.94
B5/84	0.61	0.71
B6/84	0.45	0.55

[a]The values for B3/84 were taken as unity.

common in fluorogenic branched dextrins. The number of glucosyl residues of the non-reducing-end side from the branching-point glucosyl residue and that of the reducing-end side determine the order of susceptibility. Some of these glucosyl residues are most likely recognized by the active sites of 4-α-glucanotransferases as the maltotetraosyl branch and the branching-point glucosyl residue.

Ratio of 4-α-glucanotransferase and amylo-α-1,6-glucosidase activities

Just as human liver and muscle GDEs differ from each other (*10*), porcine liver and muscle GDEs are thought to differ. GDE has two different active sites and the ratio of the 4-α-glucanotransferase to amylo-α-1,6-glucosidase activities should be characteristic to the enzyme. We thus compared the ratios of these activities of liver and muscle GDEs. Through the amylo-α-1,6-glucosidase activity of the two GDEs, fluorogenic G4PA was liberated from B3/41, and subsequently, the amount liberated increased linearly over time (data not shown). The rates of amylo-α-1,6-glucosidase action on B3/41 were thus obtained from the linear increments of G4PA. Then the ratio of the two activities for fluorogenic branched dextrins were calculated (Fig. 4). As expected, the ratio varied depending on the substrate; however, irrespective of the substrates, there were considerable differences between liver and muscle GDEs in the ratio of the two activities. These results indicate that the two enzymes are unique from each other, which is consistent with the fact that in porcine, liver and muscle GDEs are differentially expressed in a tissue-specific manner, similar to human GDEs.

Nature of brain GDE

Comparison of the ratio of the 4-α-glucanotransferase and amylo-α-1,6-glucosidase activities was effective for discriminating between liver and muscle GDEs. This method was applied to determine whether porcine brain GDE is the same as the liver or muscle counterpart, or unique from either one.

The fluorogenic branched dextrins were incubated with porcine brain GDE in the presence of G6 and analysed by HPLC, resulting in GG8PA and G8PA as fluorogenic products in all reaction mixtures (Fig. 5). The rate of the 4-α-glucanotransferase action was calculated from the total amount of GG8PA and G8PA. The ratios of the two activities were determined to be 0.155, 0.131, 0.0990 and 0.0745 for B3/84, B4/84, B5/84 and B6/84, respectively (Fig. 6).

For all substrates, the ratios for brain GDE were different from those for liver and muscle GDEs, indicating that brain GDE is unique from both liver and muscle GDEs. However, since the ratios had values between those of the liver and muscle enzymes, brain

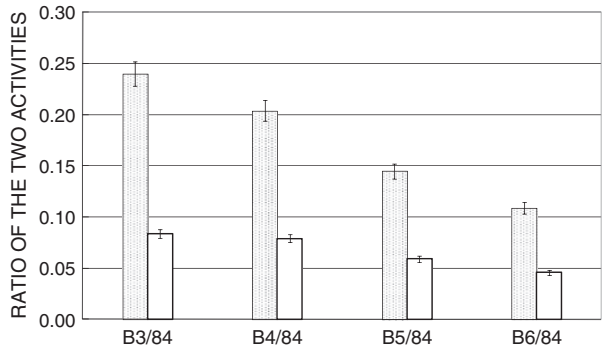

Fig. 4 Ratios of the 4-α-glucanotransferase activity to amylo-α-1, 6-glucosidase activity of porcine liver and muscle GDEs. Dotted and open columns indicate the values for liver and muscle GDEs, respectively. The values are the means of triplicate measurements.

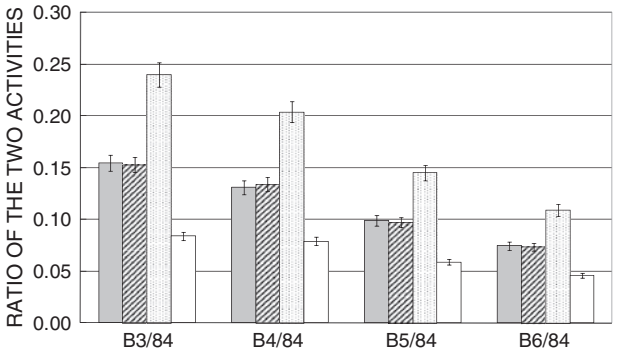

Fig. 6 Comparison of the ratio of the 4-α-glucanotransferase and amylo-α-1,6-glucosidase activities in brain, liver and muscle GDEs. Grey, hatched, dotted and open columns indicate the values for brain GDE, mixture of 45% liver and 55% muscle GDE, liver GDE and muscle GDE, respectively. The values are the means of triplicate measurements.

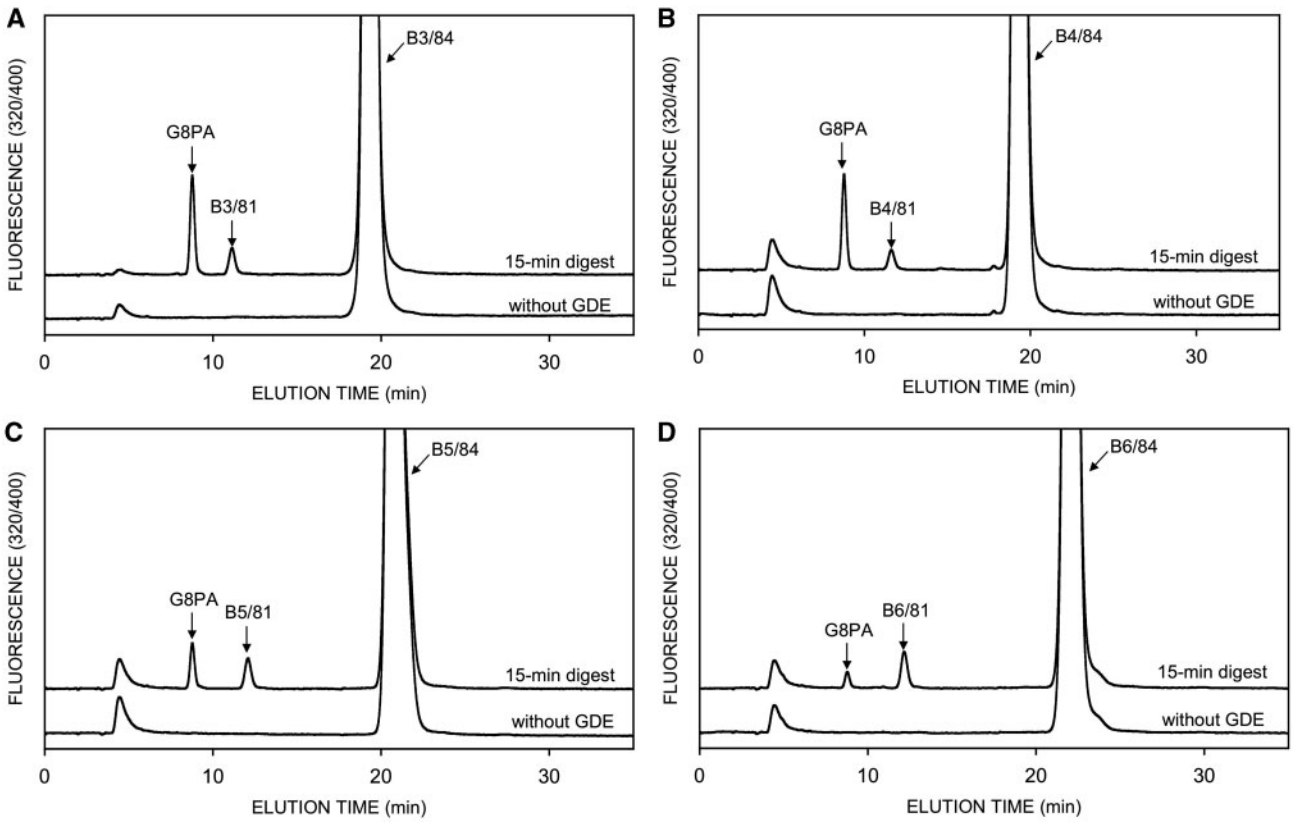

Fig. 5 HPLC analysis of fluorogenic branched dextrins digested by porcine brain GDE. Enzymatic reaction and HPLC analysis of the reaction mixture were carried out as described in 'Materials and Methods' section. The chromatograms of the reaction mixtures without GDE are shown at the bottom. (A) 15-min digest of B3/84; (B) 15-min digest of B4/84; (C) 15-min digest of B5/84; (D) 15-min digest of B6/84.

GDE might be a mixture of these two enzymes. If this is the case, the composition of brain GDE could be calculated from the set of values for liver and muscle GDEs. The percentage of liver to muscle GDE was calculated to be 45 and 55% using B3/84, 42 and 58% using B4/84, 46 and 54% using B5/84 and 45 and 55% using B6/84 (Fig. 6). Since all these values are relatively the same within a margin of error, brain GDE could comprise a mixture of 45% liver and 55% muscle GDEs. Liver and muscle GDEs are likely expressed in porcine brain and play a role in degradation of brain glycogen. On the other hand, it is not impossible that a third enzyme shows such similar values for the substrates. We cannot exclude the possibility that brain GDE is a third enzyme. Investigation of brain mRNA is needed in order to address this possibility.

Conflict of interest
None declared.

References

1. Cori, G.T. and Larner, J. (1951) Action of amylo-1, 6-glucosidase and phosphorylase on glycogen and amylopectin. *J. Biol. Chem.* **188**, 17–29
2. Larner, J. and Schliselfeld, L.H. (1956) Amylo-1,6-glucosidase. *Biochim. Biophys. Acta* **20**, 53–61
3. Gordon, R.B., Brown, D.H., and Brown, B.I. (1972) Preparation and properties of the glycogen debranching enzyme from rabbit liver. *Biochim. Biophys. Acta* **289**, 97–107
4. White, R.C. and Nelson, T.E. (1974) Re-evaluation of the subunit structure and molecular weight of rabbit muscle amylo-1,6-glucosidase/4-α-glucanotransferase. *Biochim. Biophys. Acta* **365**, 274–280
5. Taylor, C., Cox, A.J., Kernohan, J.C., and Cohen, P. (1975) Debranching enzyme from rabbit skeletal muscle, purification, properties and physiological role. *Eur. J. Biochem.* **51**, 105–115
6. Bates, E.J., Heaton, G.M., Taylor, C., Kernohan, J.C., and Cohen, P. (1975) Debranching enzyme from rabbit skeletal muscle; evidence for the location of two active centres on a single polypeptide chain. *FEBS Lett.* **58**, 181–185
7. Gillard, B.K. and Nelson, T.E. (1977) Amylo-1,6-glucosidase/4-α-glucanotransferase: use of reversible substrate model inhibitors to study the binding and active sites of rabbit muscle debranching enzyme. *Biochemistry* **16**, 3978–3987
8. Liu, W.N., Madsen, B., Braun, C., and Withers, S.G. (1991) Reassessment of the catalytic mechanism of glycogen debranching enzyme. *Biochemistry* **30**, 1419–1424
9. Nakayama, A., Yamamoto, K., and Tabata, S. (2001) Identification of the catalytic residues of bifunctional glycogen debranching enzyme. *J. Biol. Chem.* **276**, 28824–28828
10. Bao, Y., Dawson, T.L. Jr., and Chen, Y.–T. (1996) Human glycogen debranching enzyme gene (AGL): complete structural organization and characterization of the 5' flanking region. *Genomics* **38**, 155–165
11. Makino, Y. and Omichi, K. (2006) Purification of glycogen debranching enzyme from porcine brain: Evidence for glycogen catabolism in the brain. *Biosci. Biotechnol. Biochem.* **70**, 907–915
12. Newgard, C.B., Littman, D.R., van Genderen, C., Smith, M., and Fletterick, R.J. (1988) Human brain glycogen phosphorylase: cloning, sequence analysis, chromosomal mapping, tissue expression, and comparison with the human liver and muscle. *J. Biol. Chem.* **263**, 3850–3857
13. Reihart, P.H., Pfeiffer, B., Spengler, S., and Hamprecht, B. (1990) Purification of glycogen phosphorylase from bovine brain and immunocytochemical examination of rat glial primary cultures using monoclonal antibodies raised against this enzyme. *J. Neurochem.* **54**, 1474–1483
14. Watanabe, Y., Makino, Y., and Omichi, K. (2005) Fluorogenic substrates of glycogen debranching enzyme for assaying debranching activity. *Anal. Biochem.* **340**, 279–286
15. Yamamoto, E., Makino, Y., and Omichi, K. (2007) Active site mapping of amylo-α-1,6-glucosidase in porcine liver glycogen debranching enzyme using fluorogenic 6-*O*-α-glucosyl-maltooligosaccharides. *J. Biochem.* **141**, 627–634
16. Watanabe, Y., Makino, Y., and Omichi, K. (2006) Activation of 4-α-glucanotransferase activity of porcine liver glycogen debranching enzyme with cyclodextrins. *J. Biochem.* **140**, 135–140
17. Makino, Y. and Omichi, K. (2006) Acceptor specificity of 4-α-glucanotransferases of mammalian glycogen debranching enzymes. *J. Biochem.* **139**, 535–541
18. Watanabe, Y., Makino, Y., and Omichi, K. (2008) Donor substrate specificity of 4-α-glucanotransferase of porcine liver glycogen debranching enzyme and complementary action to glycogen phosphorylase on debranching. *J. Biochem.* **143**, 435–440