An Enzyme-Linked Immunosorbent Assay and Reference Ranges for Bisphosphoglycerate Mutase in Human Erythrocytes

Takayuki Takubo,^{1*} Izumi Tsuda,¹ Noriyuki Tatsumi,¹ Kenji Kasuya,² Yoshiyuki Taniguchi,² Tsuyoshi Fujita,² Kohji Uchida,² Yuhsi Matsuo,² and Taizo Hayashi³

¹Department of Clinical and Laboratory Medicine, Osaka City University Medical School, Osaka, Japan

²Nagahama Institute for Biochemical Science, Oriental Yeast Co., Ltd., Nagahama, Shiga, Japan

³Department of Clinical Pathology, Osaka Medical College, Takatsuki, Osaka, Japan

We established an enzyme-linked immunosorbent assay (ELISA) system for the determination of human bisphosphoglycerate mutase (BPGM) protein content in human erythrocytes using a polyclonal anti-BPGM antibody. We determined reference ranges for BPGM protein content, synthase activity, and specific activity in human erythrocytes. We produced a recombinant human BPGM (rBPGM) by gene manipulation using *E. coli* and then obtained the polyclonal antibody by immunizing rabbits with purified rBPGM. The reproducibility of the ELISA was in an acceptable range with a coefficient of variation under 1.5%. The ELISA was reliable in the

range of 0.1 to 10 ng/mL. The polyclonal anti-rBPGM antibody did not show any cross-reaction with recombinant human B type phosphoglycerate mutase, which is highly homologous to rBPGM. The ELISA was found to be practical for the determination of BPGM protein content in human erythrocytes. The mean BPGM protein content was 56.3 ± 9.7 $\mu\text{g/mL}$ in whole blood (mean $\pm$ SD, $n = 50$). The ELISA can be used to examine various hematologic disorders with abnormal red cell size and cell counts, and to detect BPGM enzymopathy in human erythrocytes. *J. Clin. Lab. Anal.* 12:263–267, 1998. © 1998 Wiley-Liss, Inc.

Key words: bisphosphoglycerate mutase; immunoassay; polyclonal antibody; reference range; enzymopathy

INTRODUCTION

Phosphoglycerate mutase (PGAM) is a glycolytic enzyme which catalyzes the interconversion between 2- and 3-phosphoglycerate in the presence of the cofactor, 2,3-bisphosphoglycerate (2,3-BPG), previously named 2,3-diphosphoglycerate (2,3-DPG). Bisphosphoglycerate mutase (BPGM), previously named diphosphoglycerate mutase (DPGM), is found only in human erythrocytes as homodimers of the E (erythrocyte) subunits with a molecular weight of approximately 30kDa. BPGM is a multifunctional enzyme that catalyzes both the synthesis of 2,3-BPG, an important effector of the dissociation of oxygen from hemoglobin, by mutase activity, and its degradation by phosphatase activity. Like other BPGM isozymes, PGAM exists as a dimer formed from muscle-specific (M) and nonmuscle-specific (B) subunits. The M subunit has a molecular weight of approximately 30kDa and is expressed in adult skeletal and cardiac muscle as MM homodimers. The B subunit has a molecular weight of approximately 30kDa and is expressed in adult brain, liver, kidney, and erythrocytes, where BB homodimers are formed. Both genes are expressed in the heart, where MB heterodimers are found.

Significant homology exists between PGAM-B and BPGM. Recently, Uchida et al. have produced recombinant human PGAM (rPGAM) and BPGM (rBPGM) respectively

by gene manipulation in *Escherichia coli*; they also obtained polyclonal antibodies specific for the purified rPGAM and rBPGM (4,5). The aim of our study was to establish an enzyme-linked immunosorbent assay (ELISA) system for the detection of BPGM protein content in human erythrocytes. We describe the characteristics of the immunoassay, reference ranges for BPGM and its clinical significance.

MATERIALS AND METHODS

Protein Purification

Recombinant human PGAM-B (rPGAM-B) and BPGM (rBPGM) were produced by gene manipulation using *Escherichia coli* JM109 (4,5,16,17).

Polyclonal Antibody Production

Polyclonal IgG specific to rBPGM was obtained from the antisera of immunized rabbits and prepared using the artifi-

*Correspondence to: Takayuki Takubo MD, Department of Clinical and Laboratory Medicine, Osaka City University Medical School, 1-5-7 Asahimachi, Abeno-ku, Osaka 545, Japan. E-mail: m3542254@msic.med.osaka-cu.ac.jp

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cial IgG-binding protein, pA(AB)4, and a B-type-bound column (6). The anti-rBPGM antibody was shown to possess specificity for rBPGM in human erythrocytes by Western blot analysis (15)(data not shown).

Preparation of Test Samples

Three mL of venous blood was drawn from seven healthy adult volunteers and anticoagulated with ethylenediaminetetraacetic acid dipotassium salt. After venous blood was centrifuged at 500g for 5 min, plasma was discarded, and 2 mL of physiologic saline (PS) was added to the blood cells. Cells were washed in PS three times, then resuspended in 2 mL of PS. Red blood cell counts and hemoglobin content were determined using an automated cell counter (Sysmex Toa Medical Electronics, Kobe, Japan). Washed blood cells were stored at -80°C until measurement of BPGM activity and BPGM protein content.

Preparation of the ELISA for rBPGM

Fifty μL of polyclonal antibody (10 $\mu\text{g}/\text{mL}$) specific for rBPGM was bound to microtiter plates at 4°C overnight. After being blocked with 300 μL Tris-buffered saline (50 mM Tris-HCl, 0.15M NaCl, TBS pH 7.5) containing 0.1% bovine serum albumin (BSA) at room temperature for 1 hr, plates were washed in TBS containing 0.1% Tween-20 at room temperature.

ELISA Procedure

Figure 1 shows the ELISA procedure for the determination of BPGM protein content. Fifty μL of rBPGM standard reagent or hemolysate (diluted $1:1 \times 10^4$) was added to each well and reacted with antibody for 2 hr at room temperature. Plates were washed in TBS containing 0.1% Tween-20 at room temperature; 50 μL of biotin-conjugated anti-rBPGM ($\mu\text{g}/\text{mL}$) was added and incubated for 1 hr at room temperature. After washing, 50 μL of streptavidin-labeled alkaline phosphatase (1 U/mL) was added and incubated for 1 hr at room temperature. Plates were washed, and 100 μL of chromogenic substrate solution (10 mM p-nitrophenylphosphate and 0.25M MgCl_2 in 1 M diethanolamine buffer pH 7.5) was added and reacted for 20 min at room temperature. Bound alkaline phosphatase was quantified using the alkaline phosphatase-substrate p-nitrophenylphosphate; absorbance of the reaction product was read at 415 nm with an A4 microplate reader (Tosoh, Tokyo, Japan) after the reaction was stopped by addition of 50 μL of 2N NaOH.

Assay of BPGM Activity

The determination of BPGM activity was performed according to the method previously described by Calvin et al. (3).

Fig. 1. Flow chart of the ELISA procedure for the determination of BPGM protein content.

Preparation of rBPGM Standard

rBPGM standard levels were prepared at nine concentrations of 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16, 0.08, and 0.04 ng/mL in TBS containing 0.1% BSA. The photometric density of rBPGM protein content was determined by the method described in the ELISA procedure.

Dilution Linearity

Due to freezing and dissolution, sample was diluted 1/2500 in TBS containing 0.1% BSA as a starting stock. Eleven dilutions were made: 0/10, 1/10, 2/10, 3/10, 4/10, 5/10, 6/10, 7/10, 8/10, 9/10, and 10/10 in TBS containing 0.1% BSA. The photometric density of rBPGM protein content was determined by the method as described in the ELISA procedure.

Preparation of rPGAM-B Standard to Cross-React for rBPGM

rPGAM-B standard levels were prepared at 10,000, 1000, 200, 100, 40, 20, 10, 5, 2.5, 1.25, and 0.63 ng/mL in TBS containing 0.1% BSA. The photometric density of rPGAM-B protein content was determined by the method as described in the ELISA procedure.

Preparation of rBPGM Standard for Reproducibility

rBPGM standard levels were prepared at concentrations of 0.26, 1.15, and 4.63 ng/mL. The photometric density of rPGAM-B protein content was determined as described in the ELISA procedure.

Specific Activity of BPGM

Specific activity of BPGM was calculated from the determined values of BPGM activity and BPGM protein content. Specific activity is expressed as units of synthase activity per mg of protein.

Establishment of Reference Ranges for BPGM

Venous blood (3 mL) was collected from 50 healthy adult volunteers into tubes containing dipotassium ethylenediaminetetraacetic acid. The population consisted of 37 males and 13 females, and the mean age was 36.9 years (range 21–60 years), 39.0 years in males (range 26–60 years) and 30.9 years in females (range 21–47 years).

RESULTS

Standard Testing

Standardization experiments revealed a linear relationship between the concentration of rBPGM and the optical density in the concentration range between 0 and 10 ng/mL rBPGM (Fig. 2).

Fig. 2. Cross-reactivity of a polyclonal anti-rBPGM antibody for rPGAM-B. Protein content was determined using absorbance at 415 nm. ●, rBPGM; ▲, rPGAM-B)

Dilution Testing

Linearity was obtained in the range of 0 to 12 ng/mL from 0/10 to 6/10 dilutions for rBPGM protein content (Fig. 3). From these results, an upper limit of 10 ng/mL rBPGM protein content was acceptable.

Cross-Reactivity

Cross-reaction of the polyclonal anti-rBPGM antibody was not detected with 10 µg/mL rPGAM-B (Fig. 4).

Fig. 3. Dilution linearity of BPGM-ELISA in series. Protein content was determined using absorbance at 415 nm.

Fig. 4. Standard curve of BPGM-ELISA obtained by analysis of graded amounts of purified rBPGM. Protein content was determined using absorbance at 415 nm.

Reproducibility

rBPGM protein content at three concentrations was consecutively analysed 10 times. The coefficient of variation (CV) for rBPGM protein content was consistently $\leq 1.5\%$ (Table 1).

Reference Ranges for BPGM

Reference ranges for BPGM protein content, enzyme activity, and specific activity are summarized in Table 2. The mean BPGM protein content was $56.3 \pm 9.7 \mu\text{g/mL}$ in whole blood (mean $\pm$ SD, $n = 50$). The mean BPGM enzyme activity was $1.13 \pm 0.16 \text{ U/mL}$ in whole blood, and the mean BPGM specific activity was $20.2 \pm 1.8 \text{ U/mg}$.

DISCUSSION

We developed an ELISA for the immunologic quantification of BPGM. The immunoassay is based on a polyclonal anti-

TABLE 2. Reference Ranges for BPGM Protein Content, Enzyme Activity, and Specific Activity in Human Erythrocytes From 50 Healthy Adult Volunteers^a

Sex	n	Protein content ($\mu\text{g/mL}$ whole blood)	Enzyme activity (U/mL whole blood)	Specific activity (U/mg protein)
Male	37	56.2 ± 10.2	1.13 ± 0.16	20.3 ± 1.8
Female	13	56.6 ± 8.2	1.11 ± 0.15	19.8 ± 1.6
Total	50	56.3 ± 9.7	1.13 ± 0.16	20.2 ± 1.8

^aAll ranges are reported as mean $\pm$ SD.

BPGM antibody produced by rabbits. We investigated the characteristics and usefulness of the immunoassay. In contrast to functional and biochemical methods, the ELISA allows efficient determination of a large number of test samples under routine conditions. The reproducibility of the ELISA was found to be acceptable. The test was not distorted by the presence of nonspecific proteins. The standard curve and dilution linearity indicated that BPGM protein content detected by the ELISA can be measured with sufficient accuracy in the range of 0.1 to 10 ng/mL. Furthermore, the mean specific activity of BPGM obtained from these test samples was similar to that of Calvin et al.(3). The ELISA using a polyclonal antibody is a readily available assay system for the determination of BPGM protein content in human erythrocytes.

It is well known that BPGM is an erythrocyte-specific enzyme whose main function is to synthesize 2,3-BPG, an effector for the delivery of O_2 to tissues. The first direct evidence for deficiency of 2,3-DPGM was reported by Schröter(6). The parents, sister, and grandmother of a child with severe transfusion-dependent hemolytic anemia all showed decreased red cell 2,3-DPGM (50% to 60% of normal) and decreased concentrations of 2,3-DPG (50% to 58% of normal). Travis et al. have described the clinical, laboratory, and biochemical features of the enzyme deficiency in this pedigree (1). Rosa et al. have reported the first case of a complete deficiency of erythrocyte DPGM (2). Lamarchandel et al. have investigated the genetic status of patients with complete erythrocyte BPGM deficiency (13). The assay for DPGM enzyme activity was performed according to Schröter et al. (6) and Rose et al. (10), and this enzyme activity has been studied by several authors (7–9). Although Rosa et al. purified BPGM through four chromatographies and then produced anti-BPGM antibodies (11,12), measurement of BPGM protein content has not previously been performed. Calvin et al. first reported that human BPGM could be expressed in *E. coli*, and they determined the specific activity of BPGM (3). BPGM protein content was determined according to Lowry's method (14); it was difficult to apply this determination of BPGM protein content to many samples. Our ELISA for BPGM protein content in human erythrocytes was able to determine BPGM protein content in 96 samples within 6 hr, as well as the specific activity.

TABLE 1. Reproducibility of Three Concentrations of BPGM

Sample	Recombinant human BPGM (ng/mL)		
	A	B	C
1	0.26	1.17	4.79
2	0.26	1.17	4.69
3	0.26	1.14	4.66
4	0.26	1.14	4.66
5	0.26	1.14	4.58
6	0.26	1.14	4.58
7	0.26	1.14	4.58
8	0.26	1.14	4.61
9	0.26	1.14	4.58
10	0.26	1.14	4.58
Mean $\pm$ SD	0.26 ± 0.00	1.15 ± 0.01	4.63 ± 0.07
CV (%)	0.0	1.1	1.5

We established reference ranges for BPGM protein content, enzyme activity, and specific activity in human erythrocytes. No significant difference was noted in protein content, enzyme activity, or specific activity between men and women, or according to age (18). In the future, we also plan to study the clinical usefulness of this screening test for the detection of BPGM enzymopathy and to investigate BPGM protein content and specific activity in various red cell disorders with low mean corpuscular volume, elevated reticulocyte counts, and elevated red blood cell counts.

REFERENCES

1. Travis SF, Martinez J, Gravin J Jr, Atwater J, Gillmer P: Study of a kindred with partial deficiency of red cell 2,3-diphosphoglycerate mutase (2,3-DPGM) and compensated hemolysis. *Blood* 51:1107–1116, 1978.
2. Rosa R, Prehu M-O, Beuzard Y, Rosa J: The first case of a complete deficiency of diphosphoglycerate mutase in human erythrocytes. *J Clin Invest* 62:907–915, 1978.
3. Calvin MC, Blouquit Y, Garel MC, Prehu MO, Cohen-Solal M, Rosa J, Rosa R: Human bisphosphoglycerate mutase expressed in E coli: Purification, characterization, and structure studies. *Biochimie* 72:337–343, 1990.
4. Uchida K, Kondoh K, Matsuo Y: Recombinant M-, B-, and MB-type isozymes of human phosphoglyceric acid mutase: Their large-scale production and preparation of polyclonal antibodies specific to M- and B-type isozymes. *Clin Chim Acta* 237:43–58, 1995.
5. Uchida K, Mori K, Hayashi T, Matsuo Y: Preparation of recombinant human bisphosphoglyceric acid mutase and its application. *Jpn J Clin Chem* 24(Suppl 3):75c, 1995.
6. Schröter W: Kongenitale nichtspherocytäre hämolytische anämie bei 2,3-diphosphoglyceratmutase-mangel der erythrocyten im frühen sauglingsalter. *Klin Wochenschr* 43:1147–1153, 1965.
7. Rose ZB, Liebowitz J: 2,3-diphosphoglycerate phosphatase from human erythrocytes: General properties and activation by anions. *J Biol Chem* 245:3232–3241, 1970.
8. Rose ZB: Effects of salts and pH on the rate of erythrocyte diphosphoglycerate mutase. *Arch Biochem Biophys* 158:903–909, 1973.
9. Rapoport S, Luebering J: The formation of 2,3-diphosphoglycerate in rabbit erythrocytes: The existence of a diphosphoglycerate mutase. *J Biol Chem* 183:507–516, 1950.
10. Rose ZB: The purification and properties of diphosphoglycerate mutase from human erythrocytes. *J Biol Chem* 243:4810–4820, 1968.
11. Rosa R, Gaillardon J, Rosa J: Diphosphoglycerate mutase and 2,3-diphosphoglycerate phosphatase activities of red cells: Comparative electrophoretic study. *Biochem Biophys Res Commun* 51:536–542, 1973.
12. Rosa R, Prehu M-O, Albrect-Ellmer K, Calvin MC: Partial characterization of the inactive mutant form of human red cell bisphosphoglyceromutase and comparison with alkylated form. *Biochem Biophys Acta* 742:243–249, 1983.
13. Lamarchandel V, Joulin V, Valentin C, Rosa R, Galacteros F, Rosa J, Cohen-Solal M: Compound heterozygosity in a complete erythrocyte bisphosphoglycerate mutase deficiency. *Blood* 80:2643–2649, 1992.
14. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–268, 1951.
15. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354, 1979.
16. Uchida K: Recombination and amplification of multiple portions of genomic DNA by a modified polymerase chain reaction. *Anal Biochem* 202:159–161, 1992.
17. Uchida K: cDNA encoding type B subunit of rat phosphoglycerate mutase: Its isolation and nucleotide sequence. *Arch Biochem Biophys* 288:558–561, 1991.
18. Takubo T, Tatsumi N, Tsuda I, et al.: Reference values for bisphosphoglycerate mutase protein content and specific activity in human erythrocytes. *Acta Haematol* 99:234–235, 1998.