# Molecular cloning of human glucose-6-phosphatase catalytic subunit 2 gene, and enzyme characterization

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### ABSTRACT

Human glucose-6-phosphatase ( G6Pase ) located in the endoplasmic reticulum, that catalyzes the hydrolysis of glucose-6- phosphate ( G6P ) to glucose and phosphate. There are three G6Pase genes from human : G6PC1 ( glucose-6-phosphatase catalytic subunit 1 ), G6PC2 ( glucose-6-phosphatase catalytic subunit 2 ) and G6PC3 ( glucose-6-phosphatase catalytic subunit 3 ). The three G6Pase genes expressed in different tissues, if the enzyme defect can cause different symptoms. This study focuses on G6PC2, it specifically expressed in pancreatic islets. We have commissioned the company to produce cDNA with the size of 1,068 bp that was significantly ligated with pQE30 expression vector and then transformed into E. coli Nova Blue for overexpression. The gene expressed the peptide chain G6PC2 with the molecular weight of 45 kDa. This protein was isolated by 6 His-tag can be purified by Ni-NTA column and assayed by glucose dehydrogenase-coupling method in finding activity of the enzyme. We found that wild type of G6PC2 enzyme had optimal reaction under 100 mM boric acid pH 8.0 at 37 ℃ which was catalyzing 5 mM G6P in 30 mins. According to this condition, then add several metal ion in the reaction, this enzyme is not affected by metal ions. Following the course, we selected one clone with higher level activity and three clones with lower level activity from 116 screened clones. These four clones are single-point mutation, the amino acid mutations were H78P, Y42H, Y124H, and E99K. We purified the four mutant proteins, assayed by glucose dehydrogenase-coupling method and to analyze the enzyme kinetics. The comparison with wild type G6PC2 results, in Km part : Y42H and Y124H mutants were higher than wild-type in 2.62 fold and 1.09 fold, respectively; E99K and H78P mutants were lower than wild-type in 0.61 fold and 0.65 fold, respectively; however in kcat part : Y42H and H78P mutants were higher than wild-type in 4.29 fold and 2.63 fold, respectively; Y124H and E99K mutants were lower than wild-type in 0.6 fold and 0.56 fold, respectively. We observed the amino acid alignment of alkaline phosphatase of E. coli, that has 50% similarity with G6PC2. The calibration of all the mutations in this model was tryed to indicating the possible reasons for changes in activity. H78P position in the active center of the bottom of the catalytic barrel, and proline forms a larger turn, may be related to stretch the opening of the barrel to make it easier for substrate binding to the enzyme. Y42H mutation becomes more positively charged nature, which may affect D51 and S102 to attract substrate. So that was made the substrate closer to the hydrolysis of phosphate by R166, and increased enzyme activity. E99K and Y124H have similar effects on the outcome, may be due to an increasing positive charge, when the substrate was closed to these two mutants, the phosphate easily be attracted, which led to lower the risk of hydrolysis by R166.

Keywords : glucose-6-phosphatase、glucose-6-phosphate、glucose、Enzyme kinetics

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## REFERENCES

1. Arden, S. D., Zahn, T., Steegers, S., Webb, S., Bergman, B., O'Brien, R. M., and Hutton, J. C. (1999) Molecular Cloning of a Pancreatic Islet – Specific Glucose-6-Phosphatase Catalytic Subunit – Related Protein Diabetes 48(3), 531-542 2. Bouatia-Naji, N., Rocheleau, G., Van Lommel, L., Lemaire, K., Schuit, F., Cavalcanti-Proenca, C., Marchand, M., Hartikainen, A. L., Sovio, U., De Graeve, F., Rung, J., Vaxillaire, M., Tichet, J., Marre, M., Balkau, B., Weill, J., Elliott, P., Jarvelin, M. R., Meyre, D., Polychronakos, C., Dina, C., Sladek, R., and Froguel, P. (2008) A Polymorphism Within the G6PC2 Gene Is Associated with Fasting Plasma Glucose Levels. Science 320(5879), 1085-1088. 3. Boztug, K., Appaswamy, G., Ashikov, A., Schaffer, A. A., Salzer, U., Diestelhorst, J., Germeshausen, M., Brandes, G., Lee-Gossler, J., Noyan, F., Gatzke, A. K., Minkov, M., Greil, J., Kratz, C., Petropoulou, T., Pellier, I., Bellanne-Chantelot, C., Rezaei, N., Monkemoller, K., Irani-Hakimeh, N., Bakker, H., Gerardy-Schahn, R., Zeidler, C., Grimbacher, B., Welte, K., and Klein, C. (2009) A Syndrome with Congenital Neutropenia and Mutations in G6PC3. N Engl J Med 360(1), 32-43 4. Bio-Rad Laboratories (1994) Bio-Rad Protein Assay, Bulletin No. 600-0005, Bio- Rad Laboratories GmbH, Munich, Germany. 5. Cirino P.C., Mayer K.M. and Umeno D. (2003) Generating mutant libraries using error-prone PCR. Meth in Mole Bio 231: 3-9. 6. Han, B., Serra, P., Amrani, A., Yamanouchi, J., Maree, A. F., Edelstein-Keshet, L., and Santamaria, P. (2005) Prevention of diabetes by manipulation of anti-IGRP autoimmunity: high efficiency of a low-affinity peptide. Nat Med 11(6), 645-652 7. Hutton, J. C., and O'Brien, R. M. (2009) The Glucose-6-Phosphatase Catalytic Subunit Gene Family. J Biol Chem 284(43):29241-5. 8. Laemmli U.K. (1970) Cleavage of structural protein during the assembly of the head of bacterophage T4. Nature 227: 680-685. 9. Ma, L., and Kantrowitz, E. R. (1996) Kinetic and X-Ray Structural Studies of a Mutant Escherichia coli Alkaline Phosphatase (His-412 f Gln) at One of the Zinc Binding Sites. Biochemistry 35, 2394-2402 10. Martin, C. C., Oeser, J. K., Svitek, C. A., Hunter, S. I., Hutton, J. C., and O'Brien, R. M. (2002) Identification and characterization of a human cDNA and gene encoding a ubiquitously expressed glucose-6-phosphatase catalytic subunit-related protein. J Mol Endocrinol 29, 205-222. 11. Martin, C. C., Bischof, L. J., Bergman, B., Hornbuckle, L. A., Hilliker, C., Frigeri, C., Wahl, D., Svitek, C. A., Wong, R., Goldman, J. K., Oeser, J. K., Lepretre, F., Froguel, P., O'Brien, R. M., and Hutton, J. C. (2001) Cloning and Characterization of the Human and Rat Islet-specific Glucose-6-phosphatase Catalytic Subunit-related Protein (IGRP) Genes. J Biol Chem 276(27), 25197-25207 12. Nishimura A., Morita M., Nishimura Y., and Sugino Y. (1990) A rapid and highly efficient method for preparation of competent Escherichia coli cells. Nucleic Acid Res 18, 61-69. 13. Pritchard L., Corne D., Kella D., Rowland J., Winson M. (2005) A general model of error-prone PCR. J The Bio 234: 497-509. 14. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Primer-directed enzymatic amplifi- cation of DNA with a thermostable DNA polymerase. Science 239,487-491 15. Sambrook J. and Russell D.W. (2001) Molecular cloning: a laboratory manual, third edition. Cold spring harbor laboratory press, New York. 16. Shieh, J. J., Pan, C. J., Mansfield, B. C., and Chou, J. Y. (2003) A Glucose-6-phosphate Hydrolase, Widely Expressed Outside the Liver, Can Explain Age-dependent Resolution of Hypoglycemia in Glycogen Storage Disease Type Ia. J Biol Chem 278(47), 47098-47103 17. Wang, Y., Martin, C. C., Oeser, J. K., Sarkar, S., McGuinness, O. P., Hutton, J. C., and O'Brien, R. M. (2007) Deletion of the gene encoding the islet-specific glucose-6-phosphatase catalytic subunit-related protein autoantigen results in a mild metabolic phenotype. Diabetologia 50, 774-778