UNIVERSITI PUTRA MALAYSIA

PURIFICATION, CHARACTERIZATION AND MOLECULAR STUDIES OF FRUCTOSE-6-PHOSPHATE PHOSPHOKETOLASE (F6PPK) FROM BIFIDOBACTERIA

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KHALID GHAZI DAIFALLA FANDI

Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia

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DEDICATION

This dissertation is dedicated to my beloved parents, Ghazi Al-Zoubi and Fatemah Al-Zoubi, for their constant love and inspiration. To my wife Bayan and my cute son Omar, for their love and patience in a long-distance relationship.

Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman: **Prof. Dr. Hasanah Mohd. Ghazali**

Faculty: **Food Science and Biotechnology**

Fructose-6-phosphate phosphoketolase (F6PPK; EC 4.1.2.22) is the key enzyme in the fructose-6-phosphate shunt pathway of glucose metabolism which is apparently restricted to bifidobacteria. Despite the biological importance of this bacterial group and the heterogeneity of the enzyme from different species, F6PPK in itself has not been characterized in detail with respect to size, subunit number, steady kinetics and N-terminal sequence. F6PPK was extracted and characterized for the first time from *Bifidobacterium asteroides* (isolated from the intestine of honeybees; ATTC 25909). The enzyme was purified to homogeneity using acetone fractionation at 40-70% saturation followed by fast protein liquid chromatography (FPLC) on Mono-Q anion exchange and Superose 12 gel filtration columns. The intact enzyme has a relative molecular mass of 110 ± 5 kDa as estimated by gel filtration chromatography (Sephadex G-200), and a single band was obtained on nondenaturing

PAGE. It was then shown to be that of F6PPK following elution from preparative polyacrylamid gel. Sodium dodecyl sulphate (SDS)-PAGE under nonreducing conditions revealed the presence of a single polypeptide of 110 ± 2 kDa. SDS-PAGE of F6PPK reacted with 2-mecaptoethanol revealed the presence of two polypeptides of 59 ± 1 and 53 ± 0.5 kDa, indicating a dimeric structure (α_1 β_1) with disulfide-linked subunits. The NH_2-terminal amino acid of the α chain was found to be methionine. The enzyme was stable at pH 4.5-8.0 with an optimum activity at pH 6.0. The enzyme was stable below 42°C and the optimum temperature was 30°C. The apparent K_m value of the enzyme for fructose-6-phosphate was 14.1 mM. The purified enzyme has no apparent requirement for thiamine pyrophosphate as cofactors. The enzyme was inactivated by Hg^{2+} and recovered after addition of dithiothretol, indicating that sulfhydryl group was probably involved in the enzyme activity. The features of *B. asteroides* F6PPK showed marked differences from those previously reported from animal and human strains.

F6PPK from *Bifidobacterium longum* (probiotic grade; BB536) was also purified to electrophoretic homogeneity using the same purification steps above. The purified enzyme had a molecular mass of about 300 kDa as determined by gel filtration on Superose 12. F6PPK migrated as a single electrophoretic band in non-denaturing polyacrylamide gel electrophoresis (PAGE). It is probably a tetramer containing two different subunits with molecular masses of about 93 ± 1 kDa and 59 ± 0.5 kDa, as determined by SDS-PAGE. The N-terminal amino acid sequences of the subunits were determined, and no significant similarity was found between the

deduced amino acid sequences and those in the databases of EMBL and SWISS-PORT, indicating that we may be reporting for the first time the partial sequence of F6PPK from two type strains of *Bifidobacterium* species. However, the *M*r 59 000 subunit of *B. asteroides* F6PPK showed a significant similarity (70%) with the corresponding subunit from *B. longum* species.

Oligonucleotide probes which were designed based on the deduced N-terminal amino acids sequences were unable to detect the presence of F6PPK gene using dot blot and Southern blot of the total genomic DNA from different species of bifidobacteria and other bacterial strains. In addition, the genomic library of *B. asteroides* was constructed in *Bam*HI-digested pUC19 by using about 2 to 6-kb DNA fragments obtained by partial digestion of the total genomic DNA with *Bam*HI. The transformed cells efficiency of *E. coli* XL1- blue carrying plasmids with genomic inserts was 1.1×10^4 cfu ml^{-1}, and this library may be a useful tool for fishing the gene encoding for F6PPK.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENULINAN, PENCIRIAN DAN KAJIAN MOLEKUL KE ATAS FRUKTOSA-6-FOSFAT FOSFOKETOLASE (F6PPK) DARI BIFIDOBACTERIA

Oleh

KHALID GHAZI DAIFALLA FANDI

Jun 2001

Pengerusi: **Prof. Dr. Hasanah Mohd. Gazali**

Fakulti: **Sains Makanan dan Bioteknologi**

Fruktosa-6-fosfat fosfoketolase (F6PPK; EC 4.1.2.22) merupakan enzim utama dalam lintasan penyimpangan fruktosa-6-fosfat dalam metabolisma glukosa yang terbatas kepada bifidobacteria. Walaupun kumpulan bakteria ini mempunyai kepentingan biologi yang tersendiri dan mempunyai sifat F6PPK yang berbeza dari spesis ke spesis, enzim ini masih belum pernah dicirikan secara terperinci dari segi saiz, bilangan subunit, kinetik tetap dan jujukan terminal-N. F6PPK daripada *Bifidobacterium asteroides* (dipencil dari usus lebah madu; ATCC 25909) telah diekstrak dan dicirikan buat pertama kalinya. Enzim ini telah ditulinkan sehingga homogen dengan menggunakan pemeringkatan aseton pada tahap ketepuan 40-70% diikuti dengan kromatografi cecair protein pantas (FPLC) menggunakan turus penukaran anion Mono-Q dan penurasan gel Superose 12. Enzim yang sempurna

mempunyai jisim molekul relatif sebanyak 110 ± 2 kDa menggunakan kromatografi penurasan gel (Sephadex G-200). Jalur tunggal telah diperolehi melalui PAGE ternyahasli, dan disahkan enzim F6PPK setelah protein dielusi daripada gel poliakralamid penyediaan. Dibawah keadaan ternyahasli, natrium dodesil sulfat (SDS)-PAGE telah menunjukkan kehadiran satu polipeptida berjisim 110 ± 5 kDa. SDS-PAGE ke atas F6PPK yang telah ditindakbalas dengan 2-merkaptoetanol telah menunjukkan kehadiran dua polipeptida yang masing-masing berjisim 59 ± 1 dan 53 ± 0.5 kDa. Pencerapan ini menunjukkan satu struktur dimerik (α_1 β_1) dengan subunit-subunit yang diikat dengan ikatan disulfida. Asid amino terawal di hujung NH_2 telah dikenalpasti sebagai metionin. Enzim telah didapati stabil pada pH 4.5-8.0 dengan aktiviti optimum pada pH 6.0. Enzim ini juga stabil pada suhu di bawah 42°C dan suhu optimumnya ialah 30°C. Nilai K_m nyata bagi enzim ini untuk fruktosa-6-fosfat ialah 14.1 mM. Enzim yang telah ditulinkan tidak memerlukan tiamin pirofosfat sebagai ko-faktor. Enzim ini telah ternyahaktif dalam kehadiran Hg^{2+} dan diaktifkan semula selepas penambahan ditiotretol. Pencerapan ini menunjukkan bahawa kumpulan sulfidril berkemungkinan terlibat dalam aktiviti enzim. Sifat-sifat F6PPK dari *B. asteroides* telah menunjukkan perbezaan yang ketara apabila dibandingkan dengan ciri enzim F6PPK yang diasingkan daripada haiwan dan manusia.

F6PPK daripada *Bifidobacterium longum* (gred probiotik; BB536) juga telah ditulinkan sehingga homogen secara elektroforetik dengan menggunakan langkah-langkah penulinan seperti di atas. Jisim molekul enzim ini dianggarkan sekitar

300 kDa, melalui penurasan gel dengan Superose 12. F6PPK telah bergerak sebagai satu jalur yang tunggal dalam PAGE tak-ternyahasli. Ia bermungkinan merupakan satu tetramer yang terdiri dari dua subunit yang berlainan, masing-masing berjisim sekitar 93 ± 1 dan 59 ± 0.5 kDa, seperti yang ditentukan melalui SDS-PAGE. Jujukan asid amino dari hujung N bagi subunit-subunit enzim telah ditentukan dan didapati tiada persamaan nyata diantara jujukan asid amino ini dengan jujukan asid amino lain dalam databes EMBL dan SWISS-PORT. Ini menunjukkan bahawa kami berkemungkinan melaporkan buat kali pertama jujukan sebahagian F6PPK daripada dua jenis spesis *Bifidobacterium*. Walau bagaimanapun, subunit M_r 59 000 daripada F6PPK dari *B. asteroides* menunjukkan kesamaan yang nyata (70%) dengan subunit berpadan daripada spesis *B. longum.*

Prob oligonukleotida yang telah direka berdasarkan jujukan asid amino hujung N didapati tidak mampu mengesan gen bagi F6PPK apabila pemblotan bintik dan pemblotan Southern dilakukan ke atas keseluruhan DNA genomik dari pelbagai spesis bifidobacteria dan lain-lain jenis bakteria. Sebagai tambahan, sumber data genomik bagi *B. asteroides* telah dibina dalam pUC19 terhadam-*Bam*HI dengan menggunakan sebanyak 2 hingga 6-kb pecahan DNA yang diperolehi dari penghadaman separa ke atas DNA genomik dengan enzim pembatas *Bam*HI. Kecekapan sel-sel *E. coli* XL-1 biru yang telah ditransfomasi untuk membawa plasmid yang mempunyai selitan genomik adalah 1.1×10^4 cfu ml^{-1}. Sumber data ini boleh digunakan untuk mencari gen yang mengkod F6PPK.

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LIST OF ABBREVIATIONS

Abbreviated	Full Form
Ab	Absorbance
AP	Alkaline phosphatase
APS	Ammonium Persulphate
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
°C	Degree Celsius
CFU	Colony forming unit
CIAP	Calf intestinal alkaline phosphatase
Da	Dalton
DNA	Deoxyribonucleic acid
dATP	Deoxy adenine triphoshate
dCTP	Deoxy cytidine triphosphate
dGTP	Deoxy guanidine triphosphate
dTTP	Deoxy thymidine triphospate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
E.coli	*Escherichia coli*
EtBr	Ethidium bromide
F6P	Fructose-6-phosphate
F6PPK	Fructose-6-phosphate phosphoketolase
FPLC	Fast protein liquid chromatography
g	gravity
gm	Gram
h	hour
IPTG	Isopropylthio-β-D-galactoside
Kb	Kilobase
kDa	KiloDalton
K_m	Michaelis constant
LB	Luria broth
L.M.P	Low melting piont
mg	Milligram
µg	Microgram
l	litre
ml	Millilitre
mol	mole
mm	millimeter
µM	Micromolar
M	Molar (mol/l)
Met	methionine
$MnCl_2$	Manganese chloride
MWCO	Molecular weight cut off