UNIVERSITI PUTRA MALAYSIA

DETECTION OF BIFIDOBACTERIA BREVE BY INDIRECT ENZYMELINKED IMMUNOSORBENT ASSAY (ELISA)

TEE SIEW CHOON

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By

TEE SIEW CHOON

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

August *2001*

DEDICATION

Especially dedicated to my Beloved Mum, Khor, Sister & Brothers

Abstract of this thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

DETECTION OF *BIFIDOBACTERIUM BREVE* BY INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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August 2001

Chairman: Professor Hasanah Mohd Ghazali, Ph.D.

Faculty: Food Science and Biotechnology, UPM

In this study, the suitability of fructose-6-phosphate phosphoketolase (F6PPK) from *Bifidobacterium breve,* and intact *B. breve* cells were examined as immunogens for the production of polyclonal antibodies that could be used to detect the presence of the organism. F6PPK is the enzyme that catalyzes the breakdown of fructose-6-phosphate to yield erythrose-4-phosphate and acetyl phosphate through the bifid shunt. It represents an interesting candidate as it is the key enzyme found restricted to bifidobacteria, and there are differences in molecular masses of the enzyme among human and animal sources. The study involved two stages. In the first stage, F6PPK extracted from *B. breve* was partially purified by sequential acetone-fractionation, ion-exchange chromatography and gel filtration chromatography, and then characterized. F6PPK was eluted from the anion exchanger column at 0.48 mM NaCl. Following purification by the gel filtration chromatography, the specific activity of F6PPK was found to be 11.05 fold but only 0.4% was recovered. The enzyme was not purified to homogeneity since 2 protein bands were observed on native polyacrylamide gel electrohphoresis. The apparent molecular mass of the enzyme as determined by gel filtration chromatography on Superose 12 was 200,000 Daltons. The enzyme was stable, at least for 10 minutes, between 20-50°C. The optimum temperature was 37°C. It has a pH optimum of pH 6.0.

F6PPK is not a metalloenzyme since EDTA did not reduce its activity. Cations like magnesium and calcium affected the activity of the enzyme. The thiol inhibitors *L*-cysteine and hydroxylamine HCl, were strongly inhibited activity of F6PPK by 19.8% and 17.1% respectively. The affinity constant, K_m, of F6PPK was $2x10^{-1}$ mM and maximal velocity, V_{max} was 20 µmole/min with fructose-6-phosphate as the substrate. In the second stage of this study, the production of polyclonal antibodies was achieved by injecting F6PPK obtained after gel filtration and intact cells of *B. breve* intradermally into 5 New Zealand White Rabbits, three of the rabbits were injected with F6PPK. Booster doses made in Freund Incomplete Adjuvant were administered every two weeks for a total of three times. Blood was collected once before the first injection and twice after third booster and labeled as Bleed 1, Bleed 2 and Bleed 3, respectively. Assessments of the antisera were carried out by Noncompetitive Indirect ELISA. Bleed 2 contained the highest level of antibody. Extensive checkerboard titration was performed; the optimum antiserum dilution for anti-F6PPK antiserum was 1:1600, while the anti-*B. breve* antiserum dose-response was 1:800 and conjugate antibody was used at a dilution of 1/4000. The best absorption for both antisera were obtained using phosphate buffered saline pH 7.2 as the coating buffer. Crude extract was good enough to bind to the anti-F6PPK antiserum. Both antisera were capable of detecting bifidobacteria at the species level at $1x10^5$ CFU/ml or greater. Both antisera were found to cross-react with the different strains of *B. breve*. A slight cross-reaction occurred with other *Bifidobacterium* spp. whereas they showed no significant cross-reactivity towards *Lactococcus* spp., *Lactobacillus* spp., probiotics, yogurt starter cultures and *E. coli*. ELISA described herein should allow unequivocal identification of *B. breve*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

PENGGUNAAN IKATAN-ENZIM IMMUNOSORBAN ASSEI (ELISA) SECARA TIDAK LANGSUNG UNTUK MENGESAN KEHADIRAN *BIFIDOBAKTERIUM BREVE.*

Oleh
TEE SIEW CHOON
Ogos 2001

Pengerusi: Profesor Hasanah Mohd Ghazali, Ph.D.

Fakulti: Sains Makanan dan Bioteknologi, UPM

Dalam kajian ini, kesesuaian enzim fruktosa-6-fosfat fosfoketolase (F6PPK) dari *Bifidobakterium. breve* dan sel utuh *B. breve* sebagai imunogen untuk menghasilkan antibodi poliklonal yang boleh digunakan untuk mengesan kehadiran organism tersebut telah dijalankan. F6PPK adalah enzim yang memangkin penguraian fruktosa-6-fosfat kepada eritrosa-4-fosfat dan asetil fosfat melalui "bifid shunt". F6PPK merupakan calon yang menarik kerana ia adalah enzim yang terhad kepada spesis bifidobakteria sahaja, dan juga berat molekulnya adalah berlainan bergantung kepada samada daripada sumber manusia atau haiwan. Kajian ini melibatkan dua peringkat. Pada peringkat awal, F6PPK diekstrak dari sel *B. breve* dan ditulinkan melalui pemendapan acetone, kromatografi penukaran anion dan kromatografi penurasan gel. Selepas itu, ciri-ciri enzim ditentukan. F6PPK dielusikan dari turus penukaran anion pada gradien garam yang rendah (0.48 mM NaCl). Teknik ini membaikkan darjah penulinan sebanyak 11.05 kali tetapi hanya 0.4% enzim diperolehi selepas kromatografi penurasan gel. Enzim F6PPK tidak berjaya ditulinkan secara homogenus kerana elektroforesis gel poliakrilamid semulajadi menunjukkan dua jalur protein. Berat molekul F6PPK yang ditentukan melalui kromatografi penurasan gel Superose 12 adalah 200,000 Daltons. Enzim ini didapati stabil pada 20-50°C selama 10 minit. Suhu optimumnya adalah 37°C. Enzim ini juga

stabil pada pH 6.0. F6PPK bukan sejenis metalloenzim kerana aktivitnya tidak dikurangkan oleh EDTA. Kation magnesium dan kalsium mempengaruhi aktiviti F6PPK. Penentang-penentang thiol, iaitu L-sistein dan hidroksilamin mengurangkan aktiviti enzim ini sebanyak 19.8% dan 17.1% masing-masing. Pemalar affinity K_m bagi F6PPK ialah $2x10^{-1}$ mM dan kelajuan maksimumnya V_{max} adalah 20 µmole/min menggunakan fruktosa-6-fosfat sebagai substrak. Pada peringkat kedua dalam kajian ini, penghasilan antibodi poliklonal dijalankan dengan menyuntik F6PPK yang diperolehi selepas kromatografi penurasan gel dan sel utuh *B. breve* secara intradermal ke dalam 5 New Zealand Arnab Putih. Tiga daripada arnab ini disuntik dengan F6PPK. Dos booster "Freund Imcomplete Adjunvant" disuntik sebanyak tiga kali pada setiap dua minggu. Pendarahan dilakukan sekali sebelum suntikan dilakukan dan sebanyak dua kali selepas jujukan terakhir dan dikenali sebagai Pendarahan 1, Pendarahan 2 dan Pendarahan 3. Analisis antisera dijalankan dengan melalui teknik ELISA Secara Tidak Langsung. Pendarahan 2 didapati mengandungi antibodi terbanyak. Pencairan antisera untuk anti-F6PPK antisera dan anti-B. *breve* antisera ialah 1:1600 dan 1:800 masing –masing, dan pencairan konjugat antibodi adalah 1/4000. Ekstrak kasar enzim adalah cukup baik untuk bergabung dengan anti-F6PPK antisera. Kedua-dua antisera berupaya mengesan kehadiran bifidobakteria pada peringkat spesies pada paras $1x10^5$CFU/ml atau lebih. Kedua-dua antisera juga didapati silang-reaktif dengan *B. breve* yang berlainan strain. Sedikit silang-reaktiviti berlaku terhadap spesis bifidobakteria yang lain, sementara tiada reaktif terhadap spesis-spesis dari genera *Lactobicillus*, *Lactococcus*, probiotik, kultur yogut dan *E. coli*. Teknik ELISA didapati boleh digunakan sebagai satu cara untuk mengenalpasti *B. breve* secara tepat.

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LIST OF ABBREVIATIONS

F6PPK	Fructose-6-phosphate phosphoketolase
TPY	Trypticase-phytone-yeast
spp.	species
%	percentage
mg	milligram
µg	microgram
rpm	rotation per minute
mM	millimolar
UV	ultraviolet
FPLC	Fast Protein Liquid Chromatography
EDTA	Ethylenediamine Tetraacetic Acid
PMSF	Phenyl Methyl Sulfonyl Fluoride
Native PAGE	Native polyacrylamide gel electrophoresis
MW	Molecular Weight
°C	degree Celsius
ELISA	Enzyme Linked Immunosorbant Assay
PBS	Phosphate Buffered Saline
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
CFU/ml	Colony Forming Unit per milliliter
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
IgG	Immunoglobulin G
Ag	Antigen
Ab	Antibody
PI	Isoelectric Focusing Point
GC	Gas Chromatography

1.0 INTRODUCTION

The health benefits associated with probiotic organisms have made the food industry incorporate the organisms in many fermented foods and dairy products. At the same time, the health consciousness of consumers has led to an expanding market for foods containing biocultures. One of the species currently employed in probiotic preparations is bifidobacteria.

Bifidobacteria are non-motile, non-spore-forming, gram-positive anaerobic rods of variable appearance. They are natural inhabitants of the gut of warm-blooded animals and human (Mitsuoka, 1982). Bifidobacteria are the major components of intestinal flora of healthy humans as these organisms represent 92% of the fecal flora in breast-fed human (Hori, 1983). These bacteria are considered beneficial for all age groups because they promote resistance to infection in the host (Homma, 1988).

Bifidobacterium can be isolated from different sources such as the feces or guts of human and animals, the human vagina and dental caries (Biavati *et al.*, 1992). These organisms are known as probiotics as they restrict the growth of many potential pathogens and putrefactive bacteria (Rasic, 1989). Bifidobacteria are also believed to have anticarcinogenic (Mitsuoka, 1982; Fernandes and Shahani, 1990) and anticholestrolemic (Hata *et al.*, 1982; Homma, 1988) properties. In addition, bifidobacteria are believed to improve lactose-intolerance and digestibility of milk products (Savaino and Levitt, 1985; Gilliland, 1989). It is ascribed to the synthesis of B-complex vitamins and absorption of calcium (Rasic and Kurmann, 1983; Rasic, 1989). Bifidobacteria have not been associated with infection, in fact it offers a panel of advantages for human health. They are being widely used in dairy products such

as yogurt and bifidus milks. In addition, there is considerable interest in using fermented milks containing bifidobacteria for probiotics purposes (Collins and Hall, 1984).

The genus bifidobacteria includes 32 species, of which 11 are of human origin (Tannock, 1999). At present, there are only 5 available for commercial applications (Tamime *et al.,* 1995). The most suitable species of human origin used for the production of fermented dairy products are *B. longum*, *B. breve*, *B. bifidum* and *B. infantis*. The species *B. animalis* previously isolated from warm-blooded animals have also been found in fermented dairy products (Bonoparte and Reuter, 1991; Biavati *et al.*, 1992).

The increasing number of commercial strains of bifidobacteria used in the food industry requires reliable methods for characterization and control, since it must be ensured that they are of human origin (Biavati *et al.,* 1992). Besides that, as far as human food and animal feed are concern, identifying the origin and strains of the organisms is of paramount importance because of issues to be addressed in relation to regulatory requirements.

Identification of bifidobacteria isolated from stools, commercial dairy products and other materials may be tentative or definitive. So far, *Bifidobacterium* spp. can be distinguished either among species or strains using several techniques. To date, there are several methods that have been proposed for the identification of dairy-related-bifidobacteria. Differentiation of *Bifidobacterium* spp. has mainly been performed on the basis of phenotypic characteristics such as carbohydrate

fermentation patterns and cellular morphology (Scardovi, 1986), or genotypic characteristic, and molecular approach based on DNA-DNA hybridization (Scardovi *et al.*, 1971a). In addition, Bourget *et al* (1993) has developed a method of identification using specific-specific oligosaccharide probes. Recently, specific detection of bifidobacterium strain in infant feces by gene rRNA primer probes has been developed (Kok *et al.*, 1996).

The definitive identification of a bifidobacteria strain may be accomplished with procedures such as DNA/DNA hybridization (Scardovi *et al.*, 1971a) or electrophoretic protein patterns (Biavati *et al.*, 1982). However, these methods are difficult to perform routinely in a food laboratory. DNA-DNA hybridization studies have also demonstrated that the levels of DNA relatedness between these two species (*B. breve* and *B. infantis*) are very similar (Lauer and Kandler, 1983; Bahaka *et al.*, 1993). Yamamoto *et al* (1992) also observed the oligonucleotide probes for bifidobacteria of human origin cross-reacted with a few strains of heterologous *Bifidobacterium* species of non-human origin

The elucidation of the unusual means of hexose metabolism in the bifidobacteria studied by Scardovi (1981) revealed that the enzyme, fructose-6-phosphate phosphoketolase (F6PPK), to be unique among lactose utilizing bacteria. Assay for the activity of this enzyme has become a key-differentiating test for the identification of bifidobacteria. Bifidobacteria can be distinguished from other bacterial group like lactobacilli, actinomyceae and anaerobic corynebacteria by the peculiar pathway: "fructose-6-phsophate shunt" (Scardovi, 1986) leading to the formation of lactic acid and acetic acids in the ratio 1.0:1.5 as chief end products.

However, in the case of bifidobacteria, analysis of volatile or metabolite products is of limited value since lactic and acetic acids are the main end products for all species. Scardovi *et al* (1971b) have studied the F6PPK electrophoretic mobility and demonstrated that this enzyme shows different patterns according to bifidobacteria ecology. However, *B. globusum* and *B. dentium* were shown to posses an "animal" and "human" electrophoretic type of F6PPK, respectively (Grill *et al*., 1995).

Among the various methods mentioned above, nonimmunological methods are not convenient for routine sample analyses since they are relatively costly and time-consuming and require technical expertise (Levieux and Venien, 1994). Moreover, a large data bank of various species is needed for effective protein profile comparison and DNA-DNA hybridization pattern. Immunochemical techniques provide complementary and alternative approaches to reduce the use of costly, sophisticated equipment and analysis times, while still maintaining reliability and improved sensitivity (Rosalba *et al.*, 1997).

Immunochemical techniques are potentially useful for the routine microbiological analysis of foods due to its specific nature and high sensitivity of the antibody-antigen reaction (Candlish, 1991). The interest of the immunological methods lies in their advantages of sensitivity, specificity, and cost-effectiveness. Other advantages include the simplicity of the procedures for sample preparation and the capability of routine and simultaneous analysis of large numbers of samples. The use of immunoassays by the food industry is increasing steadily. Among these Enzyme-Linked Immunosorbent Assay (ELISA) being the most widely used immunoassay in food analysis (Allen, 1990). Applications of immunological assays