UNIVERSITI PUTRA MALAYSIA

PHYTASE ACTIVITY AND ISOLATION OF THE PHYTASE GENE OF MITSUOKELLA JALALUDINII

PHANG CHIUN YEE

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PHYTASE ACTIVITY AND ISOLATION OF THE PHYTASE GENE OF *Mitsuokella jalaludinii*

By

PHANG CHIUN YEE

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

August 2008

To

My parent, my beloved husband, Che Toang, lovely son and daughter, Yu Kang and Zhi Xuan

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

PHYTASE ACTIVITY AND ISOLATION OF THE PHYTASE GENE OF *Mitsuokella jalaludinii*

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Chairman: **Professor Norhani Abdullah, PhD**

Faculty: **Institute of Bioscience**

Mitsuokella jalaludinii, a gram-negative, non-motile, non-spore-forming and rod-shaped bacterium from rumen of cattle was used in this study. The bacterium showed the ability to produce phytase enzyme indicated by with the formation of a halo when it was grown on MF1 medium containing calcium phytate after incubation at 39^{o}C for three days. The growth patterns of this bacterium in MF1 and MF1 + 0.5% Na-phytate media were similar, where the exponential phase was achieved after 6 h of incubation. The pH of the MF1 growth medium decreased from 7 to 4.96 while for MF1 + 0.5% Na-phytate medium, the pH decreased from 7 to 5.07. The phytase activity of *M. jalaludinii* was mainly present in the cell-bound fraction. The phytase activity was 4-fold higher when the bacterium was grown in MF1 + 0.5% Na-phytate medium compared to that of culture grown in MF1 medium. The phytase activity of the cell-bound fraction of culture grown in the MF1 + 0.5% Na-phytate medium was 3.1 U/ml but it was only 0.8 U/ml for the MF1 medium. The total inorganic phosphorus concentration in the MF1 + 0.5% Na-phytate medium did not inhibit phytase activity of *M. jalaludinii*.

Four pairs of PCR primers were generated based on *Selenomonas ruminantium*'s phytase gene sequence. A partial phytase gene of *M. jalaludinii* with size 736 bp was successfully isolated using PCR amplification using its genomic DNA as template. Southern hybridization showed positive signals of genomic *Pst*I fragment at sizes approximately 1.5 kb and between 4 to 5 kb by using the 736 bp clone as a probe. A size-selected genomic library at 1 to 2 kb was successfully generated. However, the phytase gene of *M. jalaludinii* was not successfully screened from the library using colony hybridization method.

DNA walking approach was used to clone the 5' end and 3'end of the phytase gene of *M. jalaludinii*. With a series of three steps of PCR amplifications, a 1.1 kb fragment was cloned and sequence. The Blastn results showed that the sequence contained part of the 5' end sequence of the phytase gene. The 3'end sequence was also successfully obtained by using the same method where a 310 bp fragment was cloned and sequenced. Primers were generated based on the sequence information of 5' end and 3' end and a 1047 bp phytase gene was isolated from *M. jalaludinii* using PCR amplification method. Phylogenetic tree study indicated that *M. jalaludinii* phytase gene was not similar to other microbial phytase genes except to that of *S. ruminantium* JY35 phytase gene and they are indeed a novel phytase.

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

AKTIVITI PHYTASE DAN PEMENCILAN PHYTASE GEN DARIPADA *Mitsuokella jalaludinii*

Oleh

PHANG CHIUN YEE

August 2008

Pengerusi: **Professor Norhani Abdullah, PhD**

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Mitsuokella jalaludinii, bakteria yang dipencilkan daripada perut lembu telah digunakan dalam kajian ini. Bakteria ini bersifat gram negatif, berbentuk rod dan tidak membentuk spora. Ia menghasilkan enzim fitase yang dapat dikesan dengan pembentukan 'halo' apabila ditumbuh dalam media MF1 yang mengandungi kalsium-fitate pada suhu 39^{o}C selama tiga hari. Corak pertumbuhan bakteria ini agak sama apabila ditumbuh dalam MF1 media dan MF1 + 0.5% Na-fitate, di mana ia mencapai tahap eksponential selepas 6 jam. pH kultur medium MF1 menurun dari nilai 7 ke 4.96 manakala pH dalam medium MF1 + 0.5% Na-fitate berkurang dari nilai 7 ke 5.07. Kebanyakan aktiviti enzim fitase dijumpai di fraksi "cell-bound". Aktiviti fitase adalah empat kali lebih tinggi apabila bacteria ditumbuhkan dalam medium MF1 + 0.5% Na-fitate berbanding dengan medium MF1. Aktiviti fitase yang dijumpai pada fraksi "cell-bound" mencapai nilai 3.1 U/ml apabila ditumbuh di dalam medium MF1 + 0.5% Na-fitate tetapi hanya mencapai nilai 0.8 U/ml apabila ditumbuh dalam medium MF1.

Empat pasang primer telah dihasilkan berdasarkan jujukan gen fitase bacteria *Selenomonas ruminantium*. Melalui kaedah PCR, sebahagian penjujukan gen fitase yang bersaiz 736 bp telah dipencilkan daripada *M. jalaludinii* menggunakan DNA genomic sebagai templat. Dengan menggunakan frakmen 736 bp sebagai prob, keputusan daripada kaedah penghibridan 'Southern' menunjukkan dua signal positif terhadap serpihan *Pst*I yang bersaiz 1.5 kb dan antara 4 hingga 5 kb. Satu perpustakaan genomik berdasarkan saiz antara 1 dan 2 kb telah dibina supaya gen fitase dapat dipencilkan. Walau bagaimanapun, gen fitase tidak berjaya dipencilkan daripada perpustakaan genomik dengan menggunakan kaedah penghibridan 'colony'.

Perjalanan DNA telah dipilih sebagai kaedah yang seterusnya untuk memencilkan hujung 5' dan hujung 3' gen fitase *M. jalaludinii*. Dengan menggunakan cara PCR tiga langkah, satu serpihan yang bersaiz 1.1 kb telah diklonkan dan dijujukkan. Keputusan Blastn menunjukkan bahawa jujukan tersebut membawa hujung 5' gen fitase. Hujung 3' gen fitase juga dipencilkan dengan menggunakan kaedah yang sama di mana satu serpihan yang bersaiz 310 bp telah diklonkan daripada genomik DNA *M. jalaludinii*. Primer-primer telah dihasilkan berdasarkan maklumat jujukan hujung 5' dan hujung 3' dan gen fitate yang besaiz 1047 kb telah berjaya dipencilkan daripada *M. jalaludinii* dengan menggunakan kaedah PCR. Analysis pokok filogenetik menunjukkan bahawa gen fitase *M. jalaludinii* adalah berbeza daripada gen fitase mikrob lain selain daripada gen fitase *S. ruminantium*. Oleh itu, kedua-dua gen fitase daripada *M. jalaludinii* dan *S. ruminantium* adalah gen fitase yang novel.

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I certify that an Examination Committee met on 6th August 2008 to conduct the final examination of Phang Chiun Yee on his degree in Master thesis entitled " Phytase Activity And Isolation of The Phytase Gene of *Mitsuokella jalaludinii*" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously and is not concurrently submitted for any other degree at UPM or at any other institution.

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PHANG CHIUN YEE

Date:

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ATCC	-	American Type Culture Collection
ATP	-	adenine triphosphate
bp	-	basepair
kbp	-	kilobasepair
BSA	-	bovine serum albumin
Ca-Phytate	-	calcium phytate
dATP	-	deoxyadenine triphosphate
dCTP	-	deoxycytosine triphosphate
dTTP	-	deoxythymine triphosphate
dGTP	-	deoxyguanine triphosphate
DNA	-	deoxyribonucleic acid
DTT	-	dithiothreitol
EDTA	-	ethylene diamine tetracetate
g	-	gram
mg	-	milligram
µg	-	microgram
HCl	-	hydrochloric acid
kDa	-	kilo Dalton
Km	-	Michaelis constant
LB	-	Luria-Bertani
LiCl	-	lithium chloride
M	-	molar / molarity
mM	-	millimolar
µM	-	micromolar
MgSO4	-	magnesium sulphate
ml	-	milliliter
µl	-	microliter
MW	-	molecular weight
N	-	Normality
NaCl	-	sodium chloride
Na-phytate	-	sodium phytate
NaOH	-	sodium hydroxide

NBT	-	nitroblue tetrazolium chloride
ng	-	nanogram
PCR	-	Polymerase Chain Reaction
pmole	-	picomole
RNA	-	ribonucleic acid
rpm	-	revolution per minute
SAAP	-	streptavidin-alkaline phosphatase conjugate
SDS	-	sodium dodecyl sulfate / sodium lauryl sulfate
SSC	-	standard saline citrate
TCA	-	trichloroacetic acid
TE	-	**T**ris-**E**DTA
Tris	-	tris[hydroxymethyl]aminomethane
Tris-HCl	-	tris hydrochloride
U	-	unit
UV	-	ultraviolet
V	-	volt
v/v	-	volume per volume
w/v	-	weight per volume
X	-	times

CHAPTER 1

INTRODUCTION

Phosphorus is an essential nutrient for all life forms. It is a very important component in nucleic acids (DNA and RNA), phospholipids and high-energy compounds (eg. ATP and GTP). The salt form, phytate or phytic acid (*myo*-inositol 1, 2, 3, 4, 5, 6 hexakiphosphate, IP6), is the main storage form of phosphorus in cereal grains, legumes, pollens and oilseeds (Pandey *et al*., 2001). These crops are grown over 90% of the world's harvested area and serve as major nutrients for humans and animals. Thus, food and feeds derived from plant sources contain large amounts of phytate.

The phosphorus in phytate is poorly utilized by monogastric animals, such as pigs, poultry, fish and humans, because they lack the enzyme which can hydrolyze the phytate, liberating the phosphorus. Therefore, inorganic phosphate has to be added to the diet to fulfill the phosphorus requirement of the animal. As a result, two main problems arise: firstly, increase in the cost of feed, and secondly, unutilized phosphorus excreted in the manure will cause phosphorus pollution of the environment. There is an alternative way to increase the phytate phosphorus utilization in these animals, i.e., by using supplemental phytase enzymes. Because of this, phytase has become an important industrial enzyme and many studies have been conducted to find new sources of the enzyme, and its production and application in the animal industry.

Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) hydrolyzes phytic acid to less phosphorylated *myo*-inositol phosphate derivatives, releasing inorganic phosphate. There are two types of phytases, namely, 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26). Both of these enzymes are classified under the family of histidine acid phosphatases (Peddington *et al*., 1993). Phytase is widespread in nature. The activity can be detected in plants, animals and a variety of microorganisms, including fungi (Shieh and Ware, 1968; Howson and Davis, 1983), bacteria (Shimizu, 1992; Greiner *et al*., 1993; Yoon *et al*., 1996), and some anaerobic ruminal bacteria (Yanke *et al*., 1998; Lan *et. al*., 2002a).

In the past few decades, techniques in molecular biology have played a major role in the production of foods and pharmaceutical compounds. With the development of cloning and heterologous microbial expression system, large amounts of enzyme can be commercially produced at a relatively low cost. Recombinant phytate-degrading enzymes from fungal species such as *Aspergillus fumigatus* (Pasamontes *et al*., 1997b; Wyss *et al*., 1998; Wyss *et al*., 1999a, b), *A. terreus* (Wyss *et al*., 1999a, b), *A. ficuum* (Ullah, 1988), *Emericella nidulans* (Wyss *et al*., 1999a, b), and the thermophilic fungus, *Thermomyces lanuginosus* (Berka *et al*., 1998), have been studied and biochemically characterized. The phytase gene of the soil fungus, *A. niger*, has been cloned and the recombinant phytase, which is known commercially as Natuphos®, has been used as a feed additive. Several bacterial phytase genes from *Bacillus subtilis* 168, *B. licheniformis* (Tye *et al*., 2002) and *Escherichia coli* (Rodriguez *et al*., 1999) have also been successfully cloned and characterized.

Ruminants, unlike monogastric animals, have the ability to utilize the phytate phosphate from feeds. Ruminants digest phytate phosphate through the action of phytase-producing bacteria residing anaerobically in the rumen (Raun *et al*., 1956). Thus, the rumen has become a target for screening phytase. Rumen bacterial species like *Selenomonas ruminantium* JY35 and *Mitsuokella jalaludinii* have been reported to produce high phytase activity (Yanke *et al*., 1998; Lan *et al*., 2002c). The phytase gene of *S. ruminantium* JY35 has been cloned and expressed into *E. coli* (Cheng. *et al*., US patent no. 5,985,605., 1999).

Mitsuokella jalaludinii is a new bacterial species that has been isolated from the rumen of local cattle (Lan *et al*., 2002a). This bacterial species produces high phytase activity (12.93 U g^{-1}) when grown in rice bran or soybean milk. Feeding trials conducted by Lan *et al.* (2002b) showed the ability of *M. jalaludinii* in improving phosphorus utilization in broilers. Thus, the enzyme has potential for industrial application. However, the bacterium requires anaerobic conditions for growth; hence mass production of the phytase enzyme would require stringent growth conditions. To overcome this problem, the phytase gene of *M. jalaludinii* could be cloned with an aerobe for phytase production. However, as mentioned above, *M. jalaludinii* is a new rumen bacterial species and therefore it is nessasary to isolate and characterize the phytase gene before it can be utilized for commercial purpose. Hence, the objectives of the present study were:

1. to confirm the presence of phytase activity of *M. jalaludinii*, and
2. to isolate and characterize the phytase gene.

CHAPTER 2

LITERATURE REVIEW

2.1 Importance of Phosphorus to the Poultry Industry and the Environmental Challenges

Phosphorus (P) is an essential component for the growth and development of all life forms. It plays important roles in skeletal structure and in vital metabolic pathways. Thus, all animals have to take sufficient amount of P in their diets. A deficiency of P in livestock diet will cause some negative effects such as bone malformation, loss of appetite and lower fertility.

For the past few decades, the poultry industry has become an important industry in livestock production. Poultry production system has changed from a backyard farming industry to an intensive large-scale industry. These changes have led to the production of large amounts of animal manure and waste within a limited area of land. In the United States, 158 million tons of dry matter livestock manure was produced per year and over 800,000 tons of nitrogen and 250,000 tons of P originated from poultry (Cromwell, 1994). In Malaysia, it has been estimated that about 37,000 tons of animal manure is produced every year (Chen, 1997) and most of it will pollute the water system. One of the pollutants from animal manure waste is P.

The P contained in feed grains and plant proteins is poorly utilized by poultry because of lack of acid phosphatase in the gut of monogastric animals (Wodzinski

and Ullah, 1996). Hence, inorganic P is added to the diet to meet the animal's requirement and unutilized P is released as manure into the environment. Environmental pollution from P in animal manure is a serious issue in areas where there is a high concentration of animals and a limited land base for waste disposal. Run-off P into the fresh water system leads to pollution of surface waters and eutrophication develops (Common, 1989; Walsh *et al*., 1994). Eutrophication is known as the main cause for the deterioration of surface water quality and disturbing the balance in the ecosystem. Thus, controlling the entry of inorganic and organic P into the water system is important to reduce environmental pollution.

2.2 Phytic Acid

2.2.1 Chemical Structure of Phytic Acid

The term "phytic acid" (*myo*-inositol-1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate, $C_6H_{18}O_{24}P_6$) is used for the free acid, the salt form of phytic acid is described as phytates, and "phytin" is for the calcium / magnesium salt. Phytic acid is a hexa-ortho-phosphate ester of *myo*-inositol. The structure of phytic acid has been derived from X-ray crystallography analysis (Blank *et al*., 1971). It consists of six phosphate groups on one six carbon molecule with a molecular weight of 659.86 (Wodzinski and Ullah, 1996). The structure of phytic acid (Figure 1) proposed by Anderson (1914) is generally accepted because this model is suitable to explain many of the physiochemical properties, interactions and nutritional effects (Sebastian *et al*., 1998).