Journal of Biochemistry and Molecular Biology, Vol. 40, No. 1, January 2007, pp. 22-28

JOURNAL OF Biochemistry and Molecular Biology

Molecular Cloning and Expression of Grass Carp *MyoD* in Yeast *Pichia pastoris*

Lixin Wang[1,2], Junjie Bai[1,*], Jianren Luo[1], Hong Chen[2], Xing Ye[1], Qing Jian[1] and Haihua Lao[1]

[1]Pearl River Fisheries Research Institute, CAFS. Key Lab. of Tropical & Subtropical Fish Breeding & Cultivation of CAFS, Guangzhou 510380, P. R. China

[2]College of Animal Science and Technology, Northwest Science and Technology University of Agriculture and Forestry, Yangling, Shaanxi 712100, P. R. China

Received 25 July 2006, Accepted 22 August 2006

MyoD, expressed in skeletal muscle lineages of vertebrate embryo, is one of muscle-specific basic helix-loop-helix (bHLH) transcription factors, which plays a key role in the determination and differentiation of all skeletal muscle lineages. In this study, a cDNA of grass carp MyoD was cloned and characterized from total RNA of grass carp embryos by RT-PCR. The full-length cDNA of grass carp MyoD is 1597 bp. The cDNA sequence analysis reveals an open reading frame of 825 bp coding for a protein of 275 amino acids, which includes a bHLH domain composed of basic domain (1-84th amino acids) and HLH domain (98-142th amino acids), without signal peptide. Then the MyoD cDNA of grass carp was cloned to yeast expression vector pPICZαA and transformed into *P. pastoris* GS115 strain, the recombinant MyoD protein with a molecular weight of about 31KD was obtained after inducing for 2d with 0.5% methanol in pH 8.0 BMGY medium, and the maximum yield was about 250 mg/L in shaking-flask fermentation. The results were expected to benefit for further studies on the crystal structure and physiological function of fish MyoD.

Keywords: Expression, Grass carp, *MyoD* gene, Molecular cloning, *Pichia pastoris*

Introduction

The MyoD was found in 1987 firstly (Davis and Weintraub, 1987), myogenin, myf5 and MRF4 were found in 1990. They were considered to be the same family (Olson, 1990). MyoD plays a key role in the determination and differentiation of all skeletal muscle lineages, and without MyoD the proliferation and differentiation of vertebrate myoblast could be affected (Braun *et al*., 1994; Sabourinm and Rudnicki, 2000; Kablar *et al*., 2003). Some researchers have proved that the number of myofibers is decided in prenatal, and increases little in postnatal (Arnold and Braun, 2000. Alves *et al*., 2003). Due to the important role in muscle development of vertebrate, the *MyoD* gene sequence, structure (Davis *et al.,* 1990; Ma *et al.,* 1994), expression in living (Sartorelli *et al*., 1994; Braun *et al.,* 1994; Kobiyama *et al.,* 1998; Zipora and Bruce, 2001), function (Braun *et al*., 1994; Sartorelli *et al*., 1994), and diagnosis (Ma *et al*., 1999; Shen *et al*., 2002) attract more and more attentions. Some recombined MyoD proteins expressed in bacteria have been reported (Maleki and Hurlburt, 1997; Chen *et al*., 2002; Yafe *et al*., 2005). However, the lack of post-translation modification in prokaryote caused the recombinant proteins existed as insoluble inclusion bodies and showed low activity. In order to carry out further study on the crystal structure and physiology function of MyoD protein, we cloned grass carp *MyoD* cDNA and constructed an engineered strain of *P. pastoris* which secretes expressed grass carp MyoD.

Materials and methods

Preparation of total RNA. The fertilized eggs of grass carp (*Ctenopharyngodon idella*) were collected from the test farm of Pearl River Fisheries Research Institute. Total RNA of grass carp embryos was extracted using SV Total RNA Isolation System (Promega), and the integrity of the RNA was analyzed through agarose gel electrophoresis.

Cloning cDNA of grass carp MyoD. 3' full RACE was conducted with the Oligo dT-Adaptor primer from the TaKaRa 5' & 3'-Full RACE core set. The 21-mer 5' oligonucleotide was 5'-ACTGCTCT (CA)GAT(C)GGCA TGATGG -3', designed according to the initial sequence homologous to the cDNA of *MyoD* of other vertebrates,

*To whom correspondence should be addressed.
Tel: 86-20-81616129; Fax: 86-20-81616162.
E-mail: baijj2005@21cn.com

such as common carp (Kobiyama *et al*., 1998), atlantic cod (Hall *et al*., 2003), rainbow trout (Rescan *et al.,* 1994), and human *MyoD* (Pearson-White *et al*., 1991). The 22-mer 3' oligonucleotide (5'-CTGATCTAGAGGTACCGGATCC-3') was from TaKaRa 3' Full-RACE Kit. The cDNA synthesis and the PCR amplification were performed according to the manual of TaKaRa 3' Full-RACE Kit. After the synthesis of single-stranded cDNA by reverse transcriptase with total poly (A) RNA as a template (30°C, 10 min; 50°C, 30 min; 95°C, 5 min; 5°C, 5 min), double-stranded cDNA was synthesized using Taq DNA polymerase subjected to 30 cycles of amplification (94°C, 30 s; 48°C, 30 s; 72°C, 2 min; 30 cycles, and then further incubated for 7 min at 72°C).

5' full RACE was conducted with the 5'-CDS Primer from the Smart™ RACE cDNA Amplification kit (BD). The 25-mer 3' oligonucleotide was 5'-GCTGTCATAACTGTTCCGTCTTCTC-3', designed according to the 3' sequence of grass carp *MyoD*. The 5' oligonucleotide from Smart™ RACE cDNA Amplification kit (BD). The cDNA synthesis and the PCR amplification were performed according to the manual of Smart™ RACE cDNA Amplification kit (BD). All the 3' and 5' RACE amplified products were electrophoresed on a 1% agarose gel, purified and reclaimed by excision from low melt point agarose gel, and cloned into the pGEM-T easy vector to be sequenced on ABI PRISMTM 377.

Construction of expression plasmid pPIC -MyoD. Three primers were used to construct the expression plasmid pPIC -MyoD, P1 (5'-CTTCCCCATCTCATCAGCTGATGAGTTCTACG-3') was designed to reconstruct the *EcoR*I site at 43rd bp of grass carp *MyoD* open reading frame(ORF) with P3 by PCR amplification; A forward primer P2 (5'-CGGAATTCATGGAGTTGTCGGATATTCCCTTCCCCATC-3') and a reverse primer P3 (5'-GCGAATTCTTAAAGAACTTGATAGATGG-3') were designed to modify the *MyoD* cDNA ORF of grass carp, including an extra *EcoR*I adapters. All the primers were designed and synthesized according to the *MyoD* cDNA of grass carp, P1 and P2 have the same 10 bp nucleotide acid sequences. After twice PCR amplifying with Pfu DNA polymerase and digesting with *EcoR*I, the *MyoD* cDNA was subcloned into the *EcoR*I site of pPICZαA. The plasmid was transformed into *E. coli* DH5α, then cultivated in low salt LB medium plates (same as LB except for a concentration of 0.5% NaCl instead of 1% NaCl) which contains 25 $\mu g \cdot ml^{-1}$ Zeocin™ (Invitrogen), positive recombinants were isolated according to the method described by Sambrook *et al.* (1989). The extracted plasmid from positive recombinant was digested with *EcoR*I and PCR amplified with α-Factor primer and *MyoD* upstream primer or downstream primer, to check the insert orientation. α-Factor primer was used for DNA sequencing.

Transformation and selection of the productive clones. The shuttle vector *Pichia pastoris* pPIC-*MyoD* and pPICZαA were transformed to the *P. pastoris* GS115 strain by LiCl method (According to EasySelect *Pichia* Expression Kit (Invitrogen)) after linearization with *Sac* I, respectively. After plating the transformants in YPDZ agar (1% yeast extract, 2% peptone, 2% glucose, 100 μg/mg Zeocin™), clones suffered homologous recombination with the AOX I sequence were selected. Then transformants were tested by PCR with *MyoD* upstream primer P2 and downstream primer P3, and inoculated to 10 ml liquid BMGY medium (1% yeast extract, 2% peptone, 100 mmol/L potassium phosphate, pH 7.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin; 1% glycerol) and shaked at 30°C till OD_{600}= 2.0. Cells were collected by centrifugation and gently resuspended in 20 ml buffer liquid BMMY medium (same as BMGY medium but containing 0.5% methanol not 1% glycerol) and cultured for another 4 days to induce expression of the recombinant protein. The recombinant production of the clones was monitored at 24, 48, 72 and 96 hours through analyzing the supernatant on SDS-15% polyacrylamide gels (described as the protocol provided by Invitrogen). The highest expression strain was designated as GS115 (pPIC-MyoD).

Optimization of recombinant protein expression. For fermentative optimization, the inductive methods, different pH value in media, final methanol concentration and induction time point were tested. The two inductive methods were that (1) According to EasySelect Pichia Expression Kit (Invitrogen), GS115 (pPIC-MyoD) was cultured to OD_{600}= 2.0 in 50 ml liquid BMGY media, transferred 5ml to 50 ml liquid BMMY media with different pH value (pH 6.5, 7.0, 7.5, 8.0, 8.5, 9.0) respectively, then added 100% methanol to a final concentration of 0.5% methanol every 24 hours to maintain induction; (2) According to the (1), GS115 (pPIC-MyoD) was cultured to OD_{600}= 2.0 in 50 ml liquid BMGY media (with optimal pH value), then transferred 5 ml to 4 medium which added 100% methanol to a final concentration of 0.25, 0.5, 0.75, 1.0% methanol, every 24 hours to maintain induction; In all of the methods, 1mL medium supernatant for SDS-PAGE analysis was aspirated from each culture every day till to 3 days. The protein lane of MyoD was scanned by gel image analysis system to calculate the percentage of MyoD in the total supernatant proteins. And the total protein concentration was determined by comparing with the protein marker.

Results

Cloning and sequence analysis. Grass carp *MyoD* cDNA 3' and 5' sequence was cloned and sequenced, the results showed that 3' sequence was 919 bp, 5' sequence was 749 bp, the full length cDNA of grass carp *MyoD* was 1597 bp. Fig. 1 showed the nucleotide sequence of grass carp *MyoD* cDNA and the deduced amino acid sequences. Grass carp *MyoD* cDNA contained a stop condon TAA, polyadenylation signal AATAAA, a poly(A) tail in 3' region. And the MyoD mature peptide contains 825 bps encoding 275 amino acids, No signal peptide was found through SignalP 3.0 analyse on line (http://www.cbs.dtu.dk/services /SignalP).

Homology and structure analysis. Comparing nucleotide and amino acid sequence of grass carp MyoD with those of other vertebrates, the results revealed that: ① the length of MyoD peptide increase from 226 amino acids (Japanese lancelt, *Branchiotoma belcheri*) to 319 amino acids (Human, *Homo sapiens*) following animal evolution from low class vertebrate to high class vertebrate, even among fish there was a difference from 267 (codfish, *Gadus morhua*) to 281 (Blue tilapia, *Oreochromis aureus*). ② The bHLH (basic helix-loop-

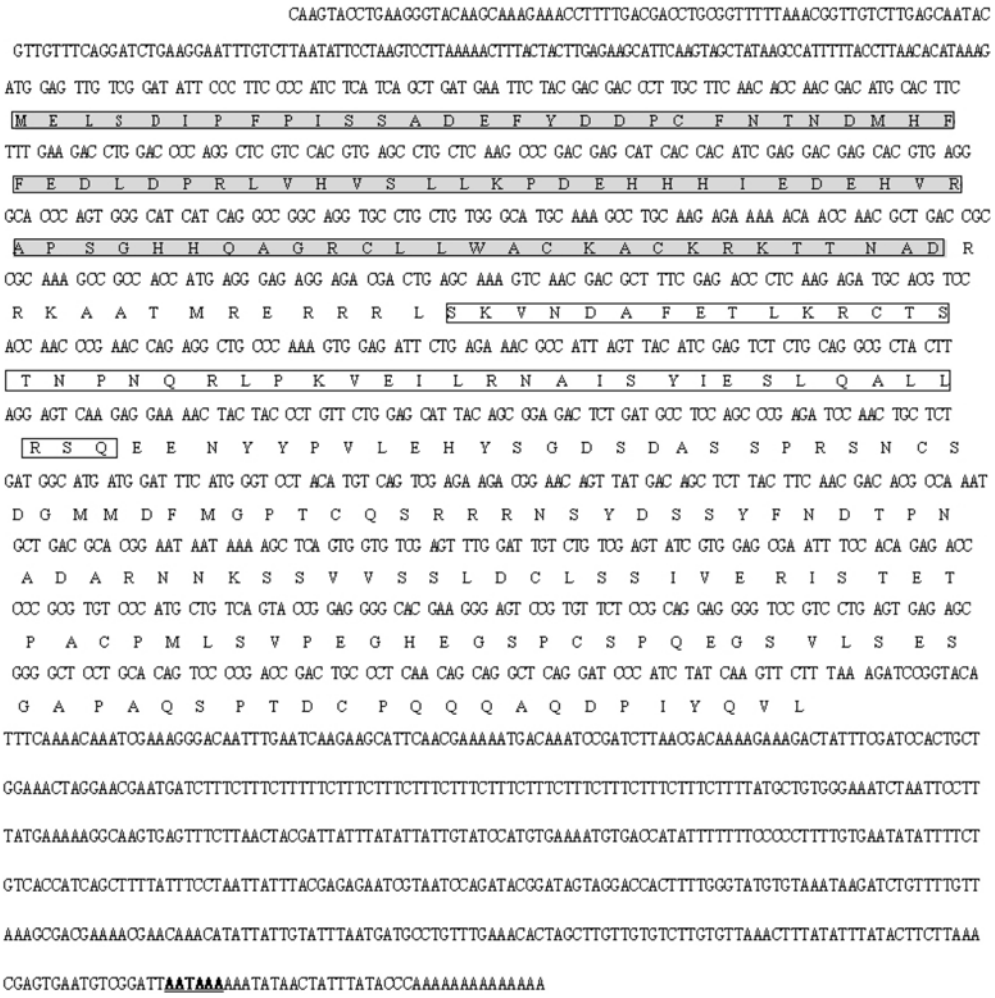

Fig. 1. Nucleotide and deduced amino acid sequences of grass carp MyoD cDNA. The 1-84th amino acids are the Basic domain of grass carp MyoD, signed ▒; The 98-142th amino acids are the HLH domain of grass carp MyoD, signed □; AATAAA signed as underline, is the polyadenylation signal.

helix, bHLH) domain of vertebrate MyoD was higher conservative than other sections, and the composition of amino acid and the length of other basic domains are quite different. Table 1 and Fig. 2 showed the comparing results of the MyoD homology and structure among vertebrates.

Construction of the MyoD expression system in *P. pastoris*. The plasmids extracted from Zeocin-resistant transformants were digested with *Eco*RI and a DNA fragment about 800 bp was obtained as expected (Fig. 3). PCR result of the transformants with α-Factor primer and *MyoD* downstream primer P3 showed a 1150 bps amplified production, including *MyoD* cDNA about 825 bp, and native vector about 350 bp. PCR result of the transformants with α-Factor primer and *MyoD* upstream primer P2 showed a 350 bp amplified production (Fig. 4). Results indicated that the inserted orientation was correct. Sequencing result revealed that the open reading frame was complete and correct, and one of the transformants was named pPIC-MyoD. After the pPIC-MyoD was linearized and transformed into *P. pastoris* strain GS115 by LiCl chemical transformation, 7 transformants were obtained on YPD Zeocin-resistant medium. The PCR result of 7 transformants with *MyoD* upstream primer P2 and downstream primer P3 showed a 800 bp DNA fragments (Fig. 5), which indicated that the recombination occurred between the plasmid and the *Pichia* genome. SDS-PAGE (Fig. 6) showed that a recombinant *MyoD* from the supernatant having a molecular weight of about 31 kD had been expressed after induced with methanol, it matched with the molecular weight of grass carp MyoD which was deduced from the amino acids by vector NT6.0. The productive clone was selected from the 7 transformants by SDS-PAGE analysis and the highest expression clone 4 was named GS115 (pPIC-MyoD) and selected for further analysis.

Optimization of recombinant protein expression. Fig. 7 showed the effect of medium pH value on recombinant MyoD expression; Fig. 8 showed the effect of methanol concentration

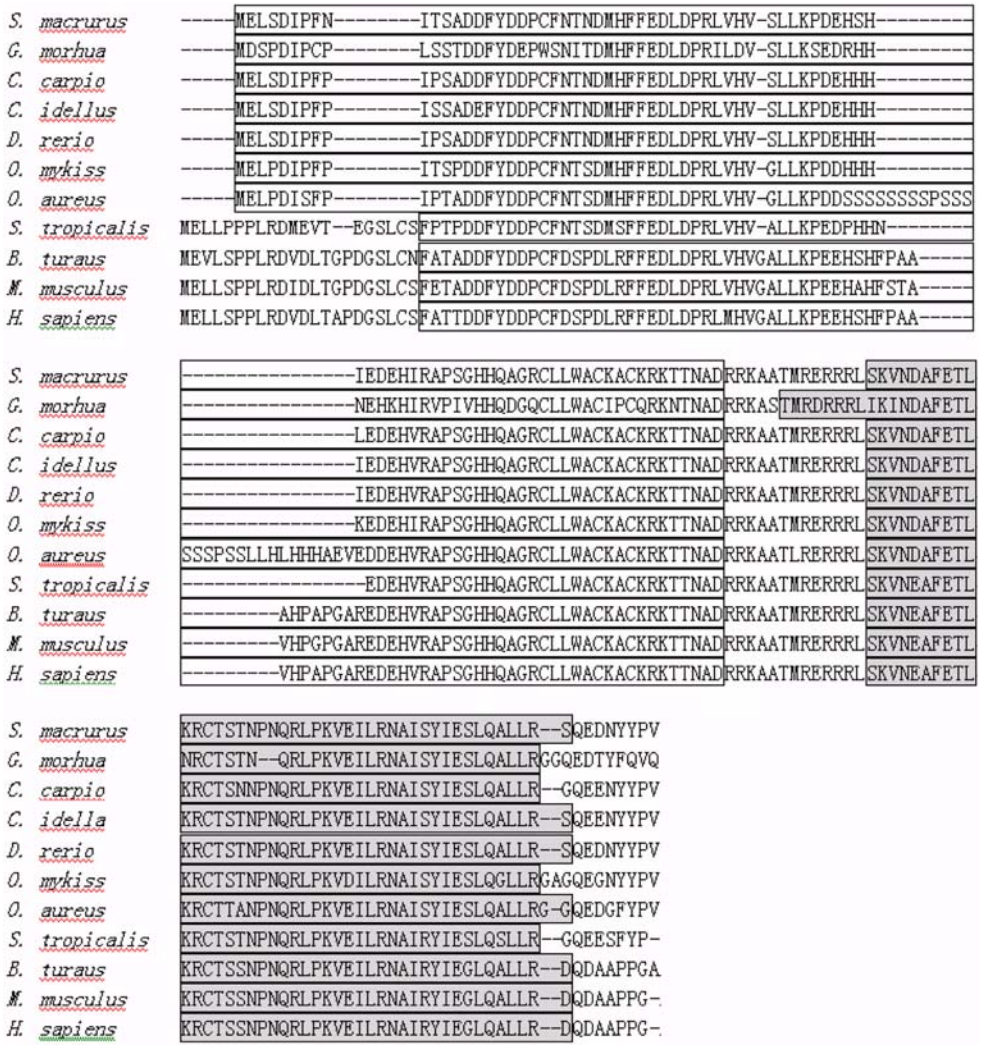

Fig. 2. Comparing the amino acid sequences and domains of MyoD protein among vertebrates. The sequence marked with □ is the basic domain; The sequence marked with ▒ is the HLH domain.

on expression; Fig. 9 showed the effect of inductive duration on expression. The optimization experiment results revealed that at 48 h post-induction, in medium (pH 8.0) and with the 0.5% methanol, the productivity of recombinant MyoD reached the highest. The SDS-PAGE gel density scanning result showed that the proportion of recombinant MyoD reached 59.8% of total supernatant proteins and total protein concentration of the supernatant was about 418 mg/L determined by comparing with the protein marker, which revealed that the maximum yield of recombinant MyoD was 250 mg per liter medium supernatant in shaking-flask fermentation.

Discussion

The grass carp *MyoD* cDNA sequence. bHLH of MRFs family includes basic domain and HLH domain, the basic domain is a site to bind the corresponding DNA, and the HLH domain interact with other relative genes. So the bHLH is conservative and an important tag to identify the MRFs family (Olson, 1990). In our research, the grass carp *MyoD* full length cDNA codes 275 amino acids, and a bHLH structure was found inside, including a basic domain located at 1-84th amino acid and a HLH domain located at 98-142th amino acid. The grass carp MyoD possess high identity with that of common carp (89.6%) and zebrafish (86.4%). No signal peptide was found by SignalP 3.0 Server. The result accorded with the character of MyoD which is expressed only in skeletal muscle cell.

The homology and characters of MyoD in vertebrate. The grass carp MyoD and that of other vertebrates were used to analyze the homology and characters of MyoD. The results showed that the HLH domains were more conservative than basic domains among different species. The basic domain of lower class vertebrates MyoD such as fish and chordate

Table 1. The homology and structure analysis of MyoD among different vertebrates

Latin name	homology of Amino acid	homology of basic and HLH Domain	Length of peptide	Length of bHLH	Location of Basic Domain	Location of HLH Domain
Branchiotoma belcheri MyoD1	36.1	40/75	226	48 + 43 = 91	31-78	93-135
Branchiotoma belcheri MyoD2	27.1	37/67	226	27 + 43 = 70	54-80	95-137
Gadus morhua	62.5	68/73	267	74 + 49 = 123	11-84	90-138
Sternopygus macrurus	89.5	96/100	274	84 + 44 = 128	1-84	98-141
Danio rerio	93.5	97/100	275	84 + 44 = 128	1-84	98-141
Cyprinus carpio	96	96/96	275	84 + 43 = 127	1-84	98-140
Ctenopharyngodon idella			275	84 + 44 = 128	1-84	98-141
Oncorhynchus. mykiss	76.0	92/94	276	84 + 43 = 127	1-84	98-140
Oreochromis aureus	62.7	68/94	281	109 + 43 = 152	1-109	123-165
Silurana tropica	68.2	81/92	288	75 + 43 = 118	21-95	109-151
Gallus gallus	71.2	70/94	298	100 + 44 = 144	1-100	114-157
Bos Taurus	55.6	71/90	318	87 + 44 = 131	23-109	123-166
Mus musculus	55.2	71/90	318	87 + 44 = 131	23-109	123-166
Homo sapiens	56.3	69/90	319	87 + 44 = 131	23-109	123-166

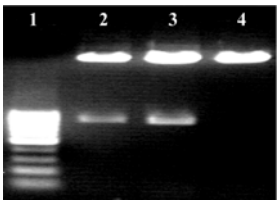

Fig. 3. Recombinant plasmids digested by *EcoR*I. 1. 100 bp Marker; 2. pPICZαA-MyoD9/*EcoR*I; 3. pPICZαA-MyoD16/*EcoR*I; 4. pPICZαA-MyoD17/*EcoR*I

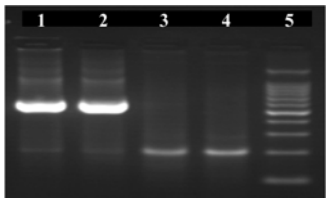

Fig. 4. PCR analysis of p PICZαA-MyoD9 and p PICZαA-MyoD16. 1-2. PCR products amplified from pPICZαA-MyoD9 and p PICZαA-MyoD16 with α-Factor primer and P3; 3-4. PCR products amplified from pPICZαA-MyoD9 and pPICZαA-MyoD16 with α-Factor primer and P2; 5. 200 bp Marker.

started at first amino acid, while it started at 21-22th amino acid in higher class vertebrates including *xenopus leavis*, cattle, mouse and human. The homology of amino acids of basic domains were different evidently (from 37% to 97%) among different species. On the contrary, HLH domain showed little difference (Table 1 and Fig. 2). The difference, which the HLH domain is more conservative than basic domain on the length and composition of amino acids, may be correlative to their functions. The bHLH domain of MyoD was regarded as the site to form heterogenic dimmer with other HLH proteins, and the activity of the heterogenic dimmer binding to the specific DNA depends on the structure of HLH and adjacency (Murre *et al*., 1989; Davis *et al*., 1990). Basic domain was considered as the specific site binding with DNA, its composition and structure can affect the binding action between MyoD and DNA, and further affected muscle differentiation (Davis *et al*., 1990; Brennan *et al*., 1991; Weintraub *et al*., 1991; Huang *et al*., 1998). It implied that the basic domain might be the key to regulate muscle development by DNA transcription.

The grass carp MyoD expression in *P. pastoris*. Expressing and getting recombined protein in vitro is a convenient way to study the crystal structure and physiological function of nature protein. However, the recombined protein that expressed in bacteria can not be folded correctly generally, due to lacking of post-translational modification which happen in the eukaryote. In order to express the grass carp MyoD and obtain recombinant protein with bioactivity effectively *in vitro*, the *Pichia pastor* expression system was used, and a grass carp MyoD high expression strain was obtained. The fermentative optimization tests showed that the optimal pH value for grass carp MyoD expression in *Pichia pastoris* was 8.0, which was different from most other genes' optimal pH value expressed in the *Pichia pastor*, such as common carp growth hormone (Li *et al.,* 2003), Chinese sturgeon cystatin (Bai *et al*., 2006), human A20 (Wu *et al*., 2006) and phytase (Kang *et al*., 2005) whose optimal pH value that expression were between pH 5.0 to pH 6.5 value. The reason that basic medium of pH 8.0 value was suitable for MyoD expression in *Pichia pastoris* may be due to the character of MyoD protein. Loewen *et al.* (1997) reported that pH value affected some genes expression in *Pichia pastoris* which contained more basic amino acids and there were 84 basic amino acids in the MyoD peptides, so basic medium may be more suitable for MyoD expression. The maximum yield of MyoD expression appeared at 2nd day

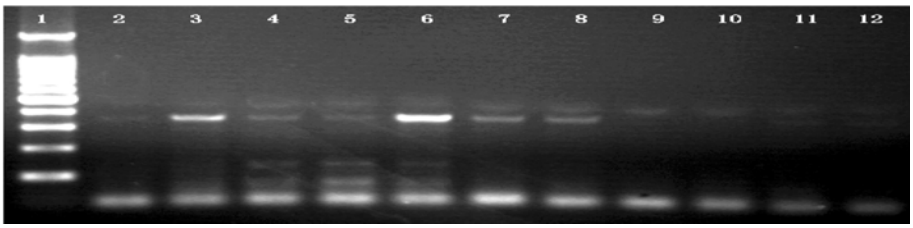

Fig. 5. Identification recombinants of *Pichia pastoris* by PCR. 1. 200 bp Marker; 2-12. PCR products of transformants amplified with P2 and P3.

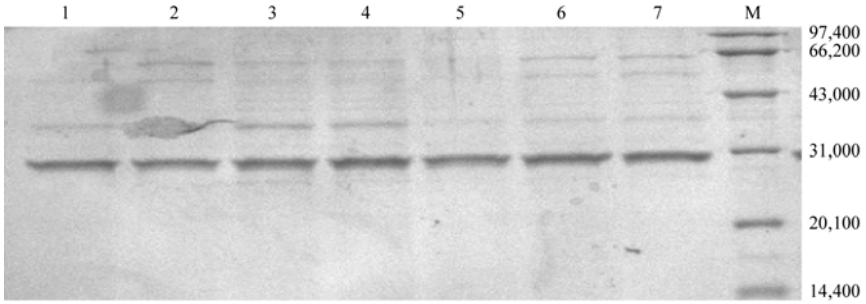

Fig. 6. SDS-PAGE detecting the MyoD protein from the supernatant of *Pichia pastoris*. M. LMW protein marker; 1-7. Protein products of GS115 supernatant (pPIC-MyoD) induced for 96 hs.

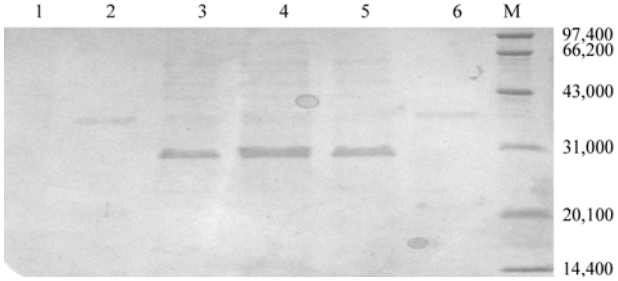

Fig. 7. The effect of pH on the expression. 1. pH 6.5; 2. pH 7.0; 3. pH 7.5; 4. pH 8.0; 5. pH 8.5; 6. pH 9.0; M. LMW protein marker.

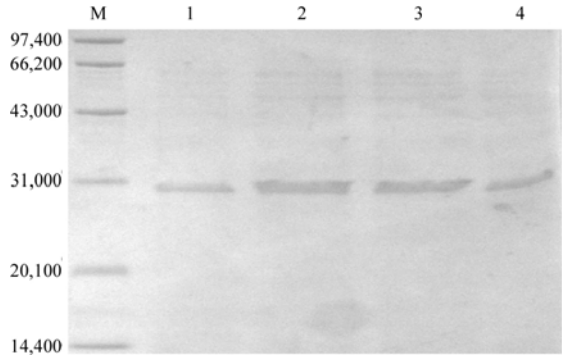

Fig. 8. The effect of methanol concentration on the expression. M. LMW protein marker; 1-4. MyoD proteins induced with methanol of 0.25%, 0.5%, 0.75% and 1.0%, respectively.

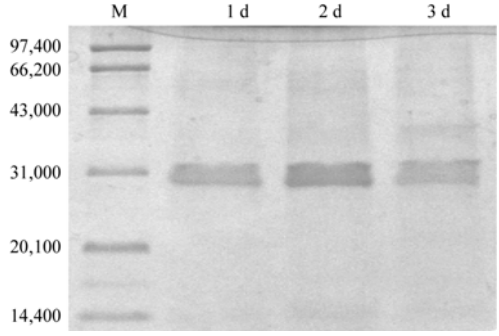

Fig. 9. The effect of inducing duration on the expression. M. LMW protein marker; 1. 1d; 2. 2d; 3. 3d.

after induced, which may also be relative to the medium pH value, for the medium pH had been dropped from 8.0 down to 7.0 after inducing for 3 days, it was not suitable for the MyoD expression in *Pichia pastoris*.

Although the MyoD protein can induce nonmuscle cells convert to the myogenic lineage efficiently (Lattanzi *et al.*, 1998; Tintignac *et al.*, 2004; Kocaefe *et al.*, 2005), we had not observed the effect of expressed MyoD on *Pichia pastoris* cell in our research, the main cause might be that the MyoD is only expressed in the vertebrate muscle cell, and yeast cell lack of a series of genes to be activated and convert into muscle cell.

As the grass carp MyoD protein has been expressed in *P. pastoris*, the research of the crystal structure and physiological function of MyoD protein will be the next work.

Acknowledgments This research work was supported by science fund of CAFS (2001-3-6) and Guangdong Province Natural Science Founding of China (021642).

References

Alves, H. J., Alvares, L. E., Gabriel, J. and Coutinho, L. L. (2003) Influence of the neural tube/notochord complex on MyoD expression and cellular proliferation in chicken embryos. *Braz. J. Med. Biol. Res.* **36**, 191-197.

Arnold, H. H. and Braun, T. (2000) Genetics of muscle determination and development. *Curr. Top. Dev. Biol.* **48**, 129-164.

Bai, J. J., Ma, D. M., Lao, H. H., Jian, Q., Ye, X., Xong, X. Y., Luo, J. R., Li, Y. H. and Liang, X. F. (2006) Molecular cloning, sequencing, expression of Chinese sturgeon cystatin in yeast Pichia pastoris and its proteinase inhibitory activity. *J. Biotechnol.* **125**, 231-241.

Braun, T., Bober, E., Rudnicki, M. A., Jaenisch, R. and Arnold, H. H. (1994) MyoD experssion marks the onset of skeletal myogenesis in Myf-5 mutant mice. *Development* **120**, 3083-3092.

Brennan, T. J., Chakraborty, T. and Olson, E. N. (1991) Mutagenesis of the myogenin basic region identifies an ancient protein motif critical for activation of myogenesis. *Proc. Natl. Acad. Sci. USA* **88**, 5675-5679.

Chen, Y. H., Liang, C. T. and Tsai, H. J. (2002) Expression, Purification and DNA-binding activity of tilapia muscle-specific transcription factor, MyoD, produced in *Escherichia coli*. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **131**, 794-805.

Davis, R. L., Weintraub, H. and Lassar, A. B. (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987-1000.

Davis, R. L., Cheng, P. F., Lassar, A. B. and Weintraub, H. (1990) The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell* **60**, 733-741.

Hall, T. E., Cole, N. J. and Johnston, I. A. (2003) Temperature and the expression of seven muscle-specific protein genes during embryogenesis in the Atlantic cod *Gadus morhua* L. *J. Exp. Biol.* **206**, 3187-3200.

Huang, J., Weintraub, H. and Kedes, L. (1998) Intramolecular regulation of MyoD activation domain conformation and function. *Mol. Cell. Biol.* **18**, 5478-5484.

Kablar, B., Krastel, K., Tajbakhsh, S. and Rudnicki, M. A. (2003) Myf5 and MyoD activation define independent myogenic compartments during embryonic development. *Dev. Biol.* **258**, 307-318.

Kocaefe, Y. C., Israeli, D., Ozguc, M., Danos, O and Garcia, L. (2005) Myogenic program induction in mature fat tissue (with MyoD expression). *Exp. Cell Res.* **308**, 300-308.

Kang, L. X., Ma, L. X. and Zhang, G. M. (2005) Inducing conditions of *Pichia pastoris* GS115/phyA producing Phytase. *J. Huazhong Agricultural University.* **24**, 477-479.

Kobiyama, A., Nihei, Y., Hirayama, Y., Kikuchi, K., Suetake, H., Johnston, I. A. and Watabe, S. (1998) Molecular cloning and developmental expression patterns of the MyoD and MEF2 families of muscle transcription factors in the carp. *J. Exp. Biol.* **201**, 2801-2813.

Li, Y. H., Bai, J. J., Jian, Q., Ye, X., Lao, H. H., Li, X. H., Luo, J. R. and Liang, X. F. (2003) Expression of common carp growth hormone in the yeast *Pichia pastoris* and growth stimulation of juvenile tilapia (*Oreochromis niloticus*). *Aquaculture* **216**, 329-341.

Loewen, M. C., Liu, X., Davies, P. L. and Daugulis, A. J. (1997) Biosynthetic production of type II fish antifreeze protein : fermentation by *Pichia pastoris*. *Appl. Microbiol. Biotechnol.* **48**, 480-486.

Lattanzi, L., Salvatori, G., Coletta, M., Sonnino, C., Cusella De Angelis, M. G., Gioglio, L., Murry, C. E., Kelly, R., Ferrari, G., Molinaro, M., Crescenzi, M., Mavilio, F. and Cossu, G. (1998) High efficiency myogenic conversion of human fibroblasts by adenoviral vector-mediated MyoD gene transfer. An alternative strategy for ex vivo gene therapy of primary myopathies. *J. Clin Invest.* **101**, 2119-2128.

Ma, P. C., Rould, M. A., Weintraub, H. and Pabo, C. O. (1994) Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. *Cell* **77**, 451-459.

Ma, X., Wang, Q. P., Wang, Z. and L., R. (1999) Expression of MyoD1 in rhabdomyosarcoma. *J. Cell Mol. Immunol.* **15**, 213-214.

Maleki, S. J. and Hurlburt, B. K. (1997) High-level expression and purification of MyoD, Myogenin, and E12. *Protein Expr. Purif.* **9**, 91-99.

Murre, C., Mc Caw, P. S. and Baltimore, D. (1989) A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* **56**, 777-783.

Olson, E. N. (1990) MyoD family: a paradigm for development. *Genes Dev.* **4**, 1454-1461.

Pearson-White, S. H. (1991) Human MyoD: cDNA and deduced amino acid sequence. *Nucleic Acids Res.* **19**, 1148.

Rescan, P. Y., Gauvry, L., Paboeuf, G. and Fauconneau, B. (1994) Identification of a muscle factor related to MyoD in a fish species. *Biochim. Biophys. Acta* **1218**, 202-204 .

Sabourin, L. A. and Rudnicki, M. A. (2000) The molecular regulation of myogenesis. *Clin. Genet.* **57**, 16-25.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: A laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York, USA.

Sartorelli, V., Puri, P. L., Hamamori, Y., Ogryzko, V., Chung, G., Nakatani, Y., Wang, J. Y. and Kedes, L. (1994) Acetylation of MyoD directed by PCAF is necessary for the execution of the muscle program. *Mol. Cell.* **4**, 725-734.

Shen, Y. G., Xu, J. G., Gu, Y. D., Hu, S. N., Huang, H. W. and Li, J. F. (2002) MyoD and Myf-5 protein expression in denervated hunan skeletal muscle. *Fudan Univ. J. Med. Sci.* **29**, 264-267.

Tintignac, L. A. J., Sirri, V., Leibovitch, M. P., Lécluse, Y., Castedo, M., Metivier, D., Kroemer, G. and Leibovitch, S. A. (2004) Mutant MyoD lacking Cdc2 phosphorylation sites delays M-phase entry. *Mol. Cell. Biol.* **24**, 1809-1821.

Weintraub, H., Dwarki, V. J., Verma, I., Davis, R., Hollenberg, S., Snider, L., Lassar, A. and Tapscott S. J. (1991) Muscle-specific transcriptional activation by MyoD. *Genes Dev.* **5**, 1377-1386.

Wu, L. J., Jiang, J. X., Zhu, P. F. and Kang, G. F. (2006) Expression of recombinant human A20 in *Pichia pastoris* GS115. *Acta Academiae Medicinae Militaris Tertiae* **28**, 201-204.

Yafe, A., Etzioni, S., Weisman, S. P. and Fry, M. (2005) Formation and properties of hairpin and tetraplex structures of guanine-rich regulatory sequences of muscle-specific gene. *Nucleic Acids Res.* **33**, 2887-2900.

Zipora, Y. R. and Bruce, M. P. (2001) MyoD and myogenin expression patterns in cultures of fetal and adult chicken myoblasts. *J. Histochem. Cytochem.* **49**, 455-462.