Molecular Cloning, Characterization, and Expression Analysis of Chicken Δ -6 Desaturase*

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ABSTRACT : Long-chain polyunsaturated fatty acids (LC-PUFA) promote the development of brain and vision of the fetus, relieve inflammation, inhibit oral dysplasia of tumor cell, decrease the incidence of cardiovascular disease and regulate arrhythmia. Δ -6 desaturase is the rate-limited enzyme in the desaturation process. This study reports the cloning, characterization and tissue expression of a Δ -6 desaturase gene in the chicken. PCR primers were designed based on the predicted sequence of chicken Δ -6 desaturase (accession number: XM421053) and used to isolate a cDNA fragment of 1,323 bp from chicken liver. Based on the 1,323 bp fragment an EST (BI390105) was obtained by BLAST. The EST and 5' end of the 1,323 bp fragment were partially overlapped. Gene specific primers derived from the EST were used for amplification of the 5' end. Another gene-specific primer derived from the 1,323 bp fragment was used for amplification of the 3' end by 3'RACE. Then the three overlapping cDNA sequences obtained were assembled with DNAMAN software and a full-length Δ -6 desaturase of 2,153 bp was obtained. The full-length cDNA contained an ORF of 1,335 bp with a 5' untranslated region of 147 nucleotides followed by an ATG initiation codon. Stop codon TGA was at position 1,481-1,483 bp. The deduced amino acids shared an homology above 77% with bovine, mice, orangutan, rat and human. The protein sequence had three histidine-rich regions HDFGH (HisI region), HFQHH (HisII region) and HH (HisIII region), a cytochrome b_5 -like domain containing a heme-binding motif and two transmembrane domains. Sequence analysis of the chicken genomic DNA revealed that the coding sequence of chicken Δ -6 desaturase included 12 exons and 11 introns. Semi-quantitative RT-PCR showed that the Δ -6 desaturase expression levels were in turn liver, spleen, pancreas, lung, breast muscle, heart, and abdominal fat. The expression of Δ -6 desaturase in liver was significantly higher than that in breast muscle ($p < 0.01$). The expression of Δ -6 desaturase in lung was significantly higher than that in abdominal fat ($p < 0.01$). This is the first clone of chicken Δ -6 desaturase. (**Key Words :** Chicken, Δ -6 desaturase, Clone, Characterization, Expression)

INTRODUCTION

Long-chain polyunsaturated fatty acids (LC-PUFA) are important components of the organism for the maintenance of normal cell function and for increasing the body's resistance (Farrell et al., 1988). They promote development

of brain and vision of the fetus, relieve inflammation, inhibit oral dysplasia of tumor cell, decrease incidence of cardiovascular disease and regulate arrhythmia. PUFA can also regulate distribution of fat and stimulate oxidation to decrease fat level. Animals can only preliminarily synthesize saturated and monounsaturated fatty acids and transform exogenous linoleic acid, α -linolenic acid, arachidonic acid into a series of PUFA by fatty acid desaturase in the body in order to meet their own needs.

Δ -6 desaturase, a rate-limiting enzyme for forming PUFA (Narce et al., 1995), catalyzes 18-C, 24-C (Andrea et al., 2002) and 16-C (Guillou et al., 2003) fatty acids to dehydrogenate at the sixth carbon atom to form a double bond. It belongs to the family of membrane desaturases. Because of its instability, it was difficult to purify and analyze the structure of desaturase. Okayasu et al. (1981) first reported the purification of Δ -9 desaturase in rat liver

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microsomes. Reddy et al. (1993) cloned the Δ -6 desaturase gene from a *Synechocystis* sp. strain producing γ -linolenic acid. These studies promoted genetic studies on Δ -6 desaturase. At present, many Δ -6 desaturase genes have been cloned from animals, plants and microbes, but information about chicken Δ -6 desaturase has not been available.

Chicken meat is the main food for consumers. The PUFA content is an important standard for measuring the quality of chicken meat. Because Δ -6 desaturase is the rate-limiting enzyme in formation of PUFA, the objectives of this study were the cloning, characterization and expression of the chicken Δ -6 desaturase gene. This should promote further study on increasing PUFA content in chicken meat through regulation of the Δ -6 desaturase gene.

MATERIALS AND METHODS

Birds

Ten 14-week-old Gushi chickens from the Henan Innovative Engineering Research Center of Poultry Germplasm Resources were used in this study. Heart, liver, lung, spleen, gizzard, pancreas, breast muscle and abdominal fat were collected from the tested chickens and immediately immersed in liquid nitrogen and then stored at -80°C before use.

Molecular cloning of Δ -6 desaturase

The total RNA was extracted from chicken liver using Redzol reagent (Beijing SBS Genetech Co.) according to the manufacturer's protocol, and was then immediately reverse-transcribed for the generation of cDNA using a first-strand cDNA synthesis kit with oligo(dT) primer (Fermentas Life Sciences) following the manufacturer's instructions.

A fragment of 1,323 bp Δ -6 desaturase cDNA was amplified by PCR using a pair of degenerate primers P1 and P2 designed on the basis of the predicted Δ -6 desaturase gene sequence (XM421053) from GenBank. PCR amplification was performed in 50 μl reaction volumes with the following protocol: 94°C for 4 min, followed by 33 cycles of 94°C for 1 min, 56.2°C for 1 min, 72°C for 2 min, and finally 72°C for 10 min. PCR products of the expected size were purified from agarose gel using a Gel Extraction Kit (Tiangen Bio Co.) and subcloned into the pUCM-T vector (Shanghai Sangon Bio Co.). Three PCR-positive colonies were selected for sequencing.

3' and 5' amplification of cDNA end

The 3' cDNA end of Δ -6 desaturase was amplified by 3' rapid amplification of cDNA ends (RACE) using 3'Full RACE Core set ver. 2.0 (Takara Bio Co). The first cDNA

for 3'RACE was obtained from the reaction of reverse transcriptase M-MLV (RNase H) (Takara Bio Co.). With heated-denatured total RNA and an oligo (dT) adaptor primer, the cDNA was amplified with the gene-specific primer P3 and 3'RACE outer primer. Nested PCR was performed with the gene-specific primer P4 and 3'RACE inner primer.

Based on the 1,323 bp fragment of Δ -6 desaturase, the EST chicken database was searched for a chicken homologue cDNA. A highly homologous chicken EST cDNA (BI390105) was obtained. The EST sequence was partly overlapped with the 5' end of the 1,323 bp fragment. By RT-PCR, a 284 bp product was amplified using the degenerate primers P5 and P6 based on the EST (BI390105).

The PCR products from 3' RACE and 5' end amplification were cloned into the pUCM-T vector (Shanghai Sangon Bio Co.) for sequencing.

Semi-quantitative RT-PCR for Δ -6 desaturase expression

Using the RedZol reagent (Beijing SBS Genetech Co.), total RNA was isolated from several tissues of chicken as described above. Genomic contamination in tissue RNA was dehydrated by DNase I (Rnase free). 4 μg of total RNA was used for reverse transcription. To compensate for the difference in loading and RT-efficiency, β -actin was used as the endogenous control. Primers (P7, P8) of Δ -6 desaturase and primers (P9, P10) of β -actin were used in semi-quantitative RT-PCR, resulting in PCR products of 238 bp and 270 bp respectively. 5 μl of PCR products were fractionated on a 1.5% agarose gel, and stained with GoldView. The fluorescence intensities with the GoldView staining were determined using the AlphaImage. The values were calculated and expressed as mean $\pm$ SD of the ratio of each PCR product to the β -actin loading control by SAS 8.12 software.

RESULTS

Cloning and characterization of Δ -6 desaturase cDNA

The full-length cDNA of Δ -6 desaturase was amplified by 3'RACE and 5' end amplification according to the scheme shown in Table 1. Using the highly degenerate primers P1 and P2, a cDNA fragment of 1,323 bp (accession number: EF636888) was obtained. A nucleotide BLAST search showed that it was homologous to Δ -6 desaturase. It shared 76.11%, 74.75%, 74.83%, 74.98% and 74.75% nucleotide homology with bovine, mouse, orangutan, rat and human, respectively. A 3'RACE product of 706 bp (accession number: EU697941) was obtained from nested PCR. Based on the EST (BI390105), a 284 bp product for the 5' end of Δ -6 desaturase was amplified. It

Figure 2. The full-length cDNA sequence and the deduced amino acids sequence of chicken Δ -6 desaturase. 5'UTR and 3'UTR are shown in lower case letters and the polyadenylation signal AATAA is in bold. Cytochrome b_5 -like domain is underlined with a single line. Three histidine-rich domains are shown in panels. Two transmembrane domains are shown by a dotted line.

Figure 3. The cytochrome b_5 -like and histidine-rich domains for chicken D6D (Δ -6 desaturase). A, a comparison of the cytochrome b_5 -like domain for chicken D6D with cytochrome b_5 (amino acids that are identical between D6D and cytochrome b_5 are highlighted in black; asterisk, two heme-binding histidines found in cytochrome b_5 -like domain); B, C, the three histidine-rich regions conserved in membrane desaturase (The histidines within these regions are identical in black, SCD is Δ -9 desaturase).

by transmembrane analysis software (http://www.ch.embnet.org/software/TMPRED_form.html) (Figure 2). Comparisons of the cytochrome b_5 -like domain with cytochrome b_5 are shown in Figure 3.

In 2004, the chicken genome map was released. The genome map was of benefit to scientists to further study the chicken Δ -6 desaturase gene. By searching NCBI, the chicken Δ -6 desaturase gene (accession number: NW_001471700) was located at the fifth chromosome. The introns and exons were obtained by contig analysis and GT-AG rule (Table 2). The coding sequence of Δ -6 desaturase included 12 exons. The first exon and intron were the longest of the exons and introns.

Analysis of Δ -6 desaturase mRNA expression

To identify functions of Δ -6 desaturase, expression of Δ -6 desaturase was firstly analyzed. Expression levels of Δ -6 desaturase mRNA in different tissues were determined by means of semi-quantitative RT-PCR. Because β -actin

mRNA levels remain fairly constant in chicken tissues regardless of their developmental or physiological condition, β -actin gene was chosen as the endogenous control in semi-quantitative RT-PCR analysis. The results showed that the levels of Δ -6 desaturase mRNA were in turn liver, spleen, pancreas, lung, breast muscle, heart, and abdominal fat. The expression of Δ -6 desaturase in liver was significantly higher than that in the breast muscle ($p < 0.01$). The difference was not significant in the liver, spleen, pancreas and lung. The expression of Δ -6 desaturase in lung was significantly higher than that in abdominal fat ($p < 0.01$). The difference was not significant in the breast muscle, heart, and abdominal fat. Expression of Δ -6 desaturase was not found in the gizzard (Figure 4).

DISCUSSION

LC-PUFA plays an important role for humans. Poultry egg is the important source of LC-PUFA and next is poultry

Table 2. The size of introns in chicken Δ -6 desaturase and the 5'splice donor and 3'splice acceptor (The exon includes initiation codon ATG and termination codon TGA except 3'UTR and 5'UTR)

Exon	Size (bp)	3' splice acceptor	5'splice donor	Intron size (bp)
1	207		CCACG/gtaag	3,374
2	111	tccag/GATGC	AAAAT/gtgag	782
3	198	tgcag/TCCCA	CCCAG/gtaag	644
4	102	ttcag/GCCCA	TTAAG/gtaaa	229
5	126	cccag/GGTGC	TTGAG/gtaag	2,003
6	61	cacag/TACGG	CCTCA/gtgag	492
7	77	tgcag/TCTTC	GGGCG/gtgag	212
8	98	cctag/GACCT	GTCAG/gtagg	2,634
9	97	tgaag/GTTTC	CTCAG/gtaat	1,137
10	80	tacag/CTGGC	CACCA/gtgag	1,103
11	126	tcaag/CCTGT	GTTGG/gtaag	526
12	52	cccag/GTCAC

Figure 4. Densitometry analysis of semi-quantitative RT-PCR. The expression of Δ -6 desaturase in liver was significantly higher than that in heart. The level of Δ -6 desaturase mRNA in lung was significantly higher than that in abdominal fat. Data are expressed as the mean $\pm$ SD (n = 10 at each tissue). ^{a, b, c} Treatments with different letters are different at $p < 0.01$.

meat. However, studies on the mechanism of metabolism of PUFA are rare. Khang et al. (2007) cloned the encoded sequence of the Δ -6 desaturase gene in Japanese quail and the homology between quail and chicken was 95.02%. The results showed that the gene was related to the concentrations of omega-6 to omega-3 PUFA.

To date there have been no reports about chicken Δ -6 desaturase and this experiment is the first study of the chicken Δ -6 desaturase gene. A 2,153 bp cDNA was obtained from the liver by RT-PCR and RACE. Its sequence was less than the predicted Δ -6 desaturase (XM421053) at the 3' end. This was probably because of one section of intron of the predicted sequence mistakenly connecting to an exon. Sequence analysis showed that there was a 1,335 bp ORF on the downstream 148-1,882 bp at the 5' end. The ORF encoded 444 amino acids and was over 77% homologous to human, rat, and mouse. The Δ -6 desaturase had three histidine-rich regions HDFGH (HisI region), HFQHH (HisII region) and HH (HisIII region), a cytochrome b_5 -like domain containing a heme-binding motif and two transmembrane domains. Zheng X (2004) reported that the cytochrome b_5 -like domain was important for the Δ -6 desaturase activity.

The coding sequence of Δ -6 desaturase included 12 exons, being similar to the human (Marquardt et al., 2000), but the number of exon base pairs was different from that in human. The first human exon was 357 bp while in the chicken it was 207 bp. The 357 bp exon of the human included 5'UTR of 150 bp. The first exon of human would also be 207 bp if the 5'UTR was removed and then the number of base pairs of the first exon of chicken and human was identical. Similarly, the 12 exons of human were found to be the same as chicken if the 5'UTR was removed. That is, the coding sequence of Δ -6 desaturase gene in chicken and human had the same exons and introns. Zheng et al.

(2005) cloned a Δ -6 desaturase gene in salmon, including 13 exons, which was different from human and chicken. However, if the UTR was removed, the coding sequence of salmon Δ -6 desaturase would have the same number of exons. With an exception that the first exon was 237 bp which was longer than chicken and salmon, the number of other base pairs was the same in chicken and human. From the results of these studies we could conclude that the coding sequence of Δ -6 desaturase is conserved in evolution.

In the human, mRNA level of Δ -6 desaturase in liver was reported to be similar to that in the lung and heart. In this experiment, mRNA in chicken liver was significantly higher than that in heart. The expression of Δ -6 desaturase in chicken heart, liver and muscle agreed with that in salmon (Zheng et al., 2005) and rainbow trout (Seiliez et al., 2001). Further studies are needed to determine if the different expression in different animals is due to differences in metabolic pathways.

In summary, this study described for the first time the cloning of chicken Δ -6 desaturase and its expression in different tissues. The level of mRNA was high in liver followed by spleen, pancreas, lung, breast muscle, heart and abdominal fat. To better understand Δ -6 desaturase, additional information such as on mechanisms of action of Δ -6 desaturase need to be further studied.

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