Molecular Cloning of αRYR Hotspot Region 1 from Broiler Chicken

Iris Lamberti Ziober¹, Fernanda Gonzales Paião¹, Silvana Regina Rockenbach Marin², Denis Fabricio Marchi¹, Eliseu Binnecke², Alexandre Lima Nepomuceno², Luiz Lehmann Coutinho³ and Massami Shimokomaki¹*

¹Departamento de Ciência e Tecnologia de Alimentos; Centro de Ciências Agrárias; Universidade Estadual de Londrina; C. P.: 6001; 86051-970; Londrina – PR – Brasil. ²Embrapa Centro Nacional de Pesquisa de Soja; 86001-970; Londrina – PR – Brasil. ³Laboratório de Biotecnologia Animal; Escola Superior de Agricultura “Luiz de Queiroz”; Universidade de São Paulo; 13418-900; Piracicaba – SP - Brasil

ABSTRACT

Samples of Pectoralis major m. were collected, and an RT-PCR analysis of the α-Ryanodine receptor (αRYR) from chicken mRNA hotspot region spanning amino acid residues 386 to 540, numbered according to the turkey sequence, revealed two classes of transcripts. The sequences of the first class were similar to turkey and human with 97% and 74% of identity, respectively, and included all transcripts with substitutions in the nucleotide sequence. The second class was characterized by the deletion of nucleotides, leading to a premature stop codon and coding for a truncated and nonfunctional protein. These results are to date the first report related to the sequencing of the chicken αRYR hotspot region 1, which will possibility serve as a guide for further studies regarding a solution in the poultry production chain related to the problem of pale, soft and exudative (PSE) meat.

Key words: αRYR, PSE, chicken

INTRODUCTION

The modern broiler is a result of genetic selection driven by the economic need to produce birds with greater muscle mass and a quicker growth rate (Barbut et al., 2008, Oda et al., 2009). However, to date little consideration has been given to examining the impact that this genetic selection for rapid growth and muscle mass gain has on muscle development. Pale, Soft and Exudative (PSE) broiler meat has been a major concern for the meat industry, and efforts have been devoted to determining its origin and developing techniques to avoid the formation of this abnormality (Olivo et al., 2001; Guarnieri et al. 2004; Barbut et al., 2008, Marchi et al., 2009; Simões et al., 2009a,b; Soares et al., 2009). PSE meat occurs due to postmortem rapid glycolysis associated with a quick pH drop while the carcass is still hot (Bendal and Wismer-Perdersen, 1962). This association between pH and high temperature causes meat protein denaturation, impairing the functional properties of muscle protein and giving rise to meat surface exudates (Candek-Potokar et al., 1998; Olivo et al., 2001). The ryanodine receptor (RyR) is a protein of approximately 5000 amino
acids that forms a large intracellular channel in the sarcoplasmic reticulum (SR) of skeletal muscle cells (Sutko and Airey, 1996). It plays a role in the rapid release of Ca$^{2+}$ from the SR during excitation-contraction (e-c) coupling (MacLennan, 2000). The RyR protein family, which includes three isoforms (RyR1, RyR2 and RyR3), is largely studied because of its importance to the pathogenic mechanisms of various diseases, such as malignant hyperthermia and central core disease in humans (Brini, 2004). In pigs, defects in RYR1 gene lead to a special condition known as Porcine Stress Syndrome (PSS) that produces PSE pork meat (Fujii et al., 1991). In turkeys, a similar problem occurs, and PSE meat is also produced (Chiang et al., 2004), although Avian Stress Syndrome symptoms still await characterization. In mammals, the major Ca$^{2+}$ release gene expressed in fast- and slow-twitch skeletal muscle is RYR1 (MacLennan, 2000), while in birds, the major genes expressed in a ratio of 1:1 are RYR1 and RYR3 (commonly called αRYR and βRYR, respectively) (Ottini et al., 1996; Sutko and Airey, 1996). A differential expression of RYR isoforms was reported in chicken skeletal muscle cells by Oda et al. (2009), who identified a relatively lower value for βRYR in animals that developed PSE compared to αRYR, suggesting this could be one possible mechanism involved in the development of broiler PSE meat. To date, no reliable genetic marker for meat quality has been defined by the poultry industry (Barbut et al., 2008); nevertheless, the study of candidate regions of RYR1 (Fig. 1) is likely to be one of the best ways to identify a marker that would provide a rapid response to selection (Barbut et al. 2008).

As seen in Figure 1, hotspot region 1 is a polypeptide subunit forming a protein structure: a large N-terminal region located at the cytoplasm. This region plays an important role in e-c coupling through its interaction with dihydropyridine receptors (DHPRs). DHPRs are L-type voltage-gated Ca$^{2+}$ channels that are present in the outer membranes of muscle cells and control the opening of RyR1 (Protasi et al., 2002).

![Figure 1 - Schematic diagram for the hotspot regions (1-3) and the predicted transmembrane domain (M1-M4) of RYR1. Adapted from Brini (2004).](image)
In mutated pig muscles, one alteration at this region, mediated by a substitution (Arg615Cys) in the RyR1 protein, causes gating hypersensitivity of the ryanodine receptors and channel openings are facilitated and closings are inhibited (Fujii et al., 1991). Ca²⁺ is released from the SR at a rate equivalent to twice that of normal muscle (Cheah and Cheah, 1976). The physiological effect of this mutation on RYR1 is an elevation of Ca²⁺ levels in resting muscle. It has been shown in pig that an elevated resting Ca²⁺ level before slaughter promotes muscle hypermetabolism and leads to muscle heat production and acidosis. The combination of these two factors causes postmortem protein denaturation and a significant deterioration in meat quality. Although this fact is well known in pigs, the genetic basis of PSE in poultry is not currently well understood. The aim of this work was to sequence and analyze the broiler chicken species (Gallus gallus) hotspot region 1 of αRYR mRNA.

MATERIALS AND METHODS

Seventy-five 42-day-old birds from a commercial line were manually slaughtered under laboratory conditions by cutting the carotid artery and jugular vein without previous electrical stunning. Just after bleeding, muscle samples (0.5 x 2.0 x 1.0 cm³) were collected and stored in microtubes (1.5 mL), rapidly frozen in liquid nitrogen and stored at –80°C. Total RNA was extracted from Pectoralis major m. based on the method developed by Chomczynski and Sacchi (1987). The cDNA was obtained through reverse transcription with SuperScript III RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA), following the manufacturer’s instructions.

For PCR amplification, the primers were the same used by Chiang et al. (2004) for turkey (Meleagris gallopavo), with the following sequences: 5’-CTG CAC CAG GAG GGC CAC ATG GAC GA-3’ (forward) and 5’-CGG TCC AGT TTG CTG ACC AGC CAG TCC AGG-3’ (reverse). The PCR amplification consisted of an initial denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, 64.5°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 8 min. PCR confirmation was conducted by agarose gel electrophoresis, and the amplified fragments were cut from the gel and purified using the PureLink™ Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. All PCR products were inserted into the TOPO TA Cloning® vector (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions and transformed using DH5α electrocompe...
The hotspot region 1 of the identified chicken αRYR showed 94% homology to the same region in the turkey sequence, 74% to some mammalian species (pig, horse, dog, rabbit) and 72% to the human sequence, according to the BLAST software (Basic Local Alignment Sequence Tool) (Altschul et al., 1990).

All 75 samples presented high similarity to each other, and the most common and frequent sequence was deposited at NCBI GenBank (GQ337080). Sixty polymorphisms were detected in the transcript variants. Comparing the new sequence of chicken αRYR (hotspot 1) in an alignment of the protein between different species, a high degree of homology was observed. These 155 amino acid residues, obtained from translation of chicken cDNA amplified in this work, were highly conserved compared to turkey αRyR (97% homology), but were less conserved when compared to RyR1s of mammalian species (Fig. 3).

All 75 chicken αRYR transcript sequences belonged to the pfam 01365 domain, which is called the RIH (RyR and IP3R Homology) domain. This is an extracellular domain, which can be part of two types of calcium channels: the RyR and the inositol 1,4,5-trisphosphate receptor (IP3) (Sanger Institute, 2009).

So far, to our knowledge, this is the first report of substitutions or deletions in the hotspot region 1 of the chicken αRYR gene.

In order to verify whether some of the alterations within the nucleotide and amino acid sequences would cause changes at the protein structure level, the SIFT (Sorting Intolerant From Tolerant - http://sift.jcvi.org/) program was used. This program can predict whether the substitution of one amino acid will affect the protein function based on the sequence similarity and the amino acids physicochemical properties.

The Fig. 4 shows the variation of amino acid substitutions and different transcript variants from chicken *Pectoralis major* m. In all transcript variants shown in Fig. 3, the amino acid substitutions are tolerated, according to the SIFT program. However, we found some transcripts that had one or more amino acid substitutions that would lead to probable changes within the protein function.

Chiang et al. (2004) also found three transcript variants in turkeys; two of them had deletions in the nucleotide sequence, which led to a deletion in exons, and the third had the normal sequence.

The substitutions or deletions in the nucleotide sequence of RYR similar to those reported in this experiment were also described by Chiang et al. (2007); however, these changes were observed in the turkey βRYR sequence. These alterations were characterized as SNPs (Single Nucleotide Polymorphisms), although any of the SNPs led to alterations in the primary structure of the protein.
The transcript GD269030 and others that had a nucleotide deletion, which created a premature stop codon, resulted in a truncated protein. However, the mRNAs that would produce truncated proteins are degraded by the NMD (Nonsense-mediated mRNA decay) pathway (Chang et al., 2007).

Figure 3 - Alignment of chicken αRyR amino acid sequence with published skeletal muscle RyR α and 1 isoforms from different species.

Figure 4 - Partial global alignment of the RyR1 protein from some of the new transcripts. Amino acid changes are in bold.

This pathway is able to detect the mRNAs that have premature termination codons (UAA, UAG, UGA), and these nonsense mRNAs are degraded and no protein is produced (Culbertson, 1999; Chang et al., 2007). Yet, this pathway does not seem to work when the mutations occur at the 3’ end of the gene (Culbertson, 1999).

The alterations observed in this study do not correlate with those found by Fujii et al. (1991) in pigs. In sensitive pigs, there is an exchange of amino acid 615 (an arginine to a cysteine) due to a point mutation in the cDNA on nucleotide #1843, where a cytosine is replaced by a thymine, leading to PSE. The animals with this mutation are prone to develop PSS and consequently PSE meat (Fujii et al. 1991).

Therefore, it can be concluded that the amplified region of 468 bp, as part of a total αRyR gene of...
approximately 15,000 bp, could serve as a guide for further and directed studies towards the solution of abnormalities in the poultry meat chain. As has been previously reported, meat quality problems such as PSE have been strongly correlated to this gene in pigs and turkeys (Fujii et al., 1991; Chiang et al., 2004).

ACKNOWLEDGEMENTS

This work was funded by CNPq and Fundação Araucária/CNPq Pronex (Protocol # 09.277) and Fundação Araucária/Finep under the BioAgroPar Program. We would also like to thank CNPq and the CAPES Foundation for a graduate scholarship to ILZ and DFM, respectively. ALN, LLC and MS are CNPq Research Fellows. FGP is under a Post-Doctorate CNPq Scholarship Program (Process CNPq 151809/2008-9).

REFERENCES

Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. (1990), Basic local alignment search tool. *J Mol Biol.* **215**, 403-410


RESUMO

Amostras do músculo *Pectoralis major* foram coletadas e uma RT-PCR foi conduzida para avaliar a sequência do mRNA do αRYR, região compreendida entre os resíduos de aminoácido 386-540, numerado de acordo com a sequência de perus. Os resultados revelaram duas classes de transcritos. O primeiro teve 97% e 74% de identidade com as sequências de αRyR e RyR1 de perus e humanos, respectivamente, e incluiu todos os transcritos com substituições de nucleotídeos. A segunda classe de transcritos foi caracterizada pela deleção de bases que levaram a um stop códon prematuro e a uma proteína truncada não-funcional. Esses resultados são até o momento, o primeiro relato de sequenciamento do αRYR, região hotspot 1 de frangos e podem servir como guia para estudos futuros na tentativa de se encontrar uma solução para os problemas na cadeia de produção de frangos relacionados com as carnes PSE (pálida, flácida e exsudativa).


Página
Em
Branco