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Prokaryotic expression, purification of chicken calpastatin protein and production of calpastatin polyclonal antibody*

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The open reading frame of chicken calpastatin (*CAST*) gene composed of 2,301 base pairs was ligated into a prokaryotic expression vector pET21a (+) to yield pET21a - CAST. The C-terminal His-tagged CAST protein was then expressed in *E. coli.* BL21 (DE3). SDS-PAGE analysis confirmed the successful expression of the fusion protein following induction with isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant protein consisted of 776 amino acid residues with an apparent molecular weight of approximately 110 kDa. It was primarily expressed as a soluble protein with a heat-stable feature. After being purified by Ni^{2+}-NTA affinity resin, a polyclonal antibody was raised against the purified His-tagged CAST protein in rabbits. The reactivity and specificity of the polyclonal antibody were both subsequently characterized by ELISA. The study provides an important experimental tool for further research on the quantification of chicken CAST protein.

KEY WORDS: calpastatin / chicken / gene / polyclonal antibody / prokaryotic expression

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Calpastatin (CAST) is a specific endogenous inhibitor of the ubiquitous calcium-activated neutral protease (CANP) and a major modulator of the metabolic turnover of cellular proteins. In human, CAST is involved in the atrophy of skeletal muscles [Enns *et al.* 2007] and systemic rheumatic diseases [Kanazawa *et al.* 2000]. In domestic animals, *CAST* gene is widely studied as a candidate gene that affects animal performance and plays a key role in the process of meat tenderization *post-mortem*. Favourable *CAST* alleles were found to correlate with increased meat tenderness and therefore had beneficial effects on its eating quality [Chung and Davis 2012, Schenkel *et al.* 2006, Casas *et al.* 2006, Nonneman *et al.* 2011, Lindholm-Perry *et al.* 2009, Gandolfi *et al.* 2011, Byun *et al.* 2008]. Despite all the available information concerning genetic variants in *CAST* gene, little is known about how this gene is regulated at the translational level, especially in chicken. Up till now, we are aware of only limited studies on chicken as to the identification of SNPs on chicken *CAST* gene and mRNA expression profiles in various tissues [Zhang *et al.* 2012] with no reports on the quantification of CAST protein in muscle tissues.

The objective of this study was to develop an efficient protocol for the production of chicken CAST protein with high purity and yield rate, and then establish a convenient approach to quantify the abundance of CAST protein by a direct ELISA assay. The result will make it possible to quantify the expression of *CAST* gene at the translational level.

Material and methods

Construction of the plasmid expressing the His-tagged CAST protein

The complete mRNA sequence of chicken *CAST* gene (GI: 212549669) was initially obtained from GenBank (http://www.ncbi.nlm.nih.gov). The chicken CAST open-reading frame (ORF) was synthesized (TAIHE Biotechnology Co., LTD., Beijing, China) and inserted into the prokaryotic expression vector pET21a (+) (NOVAGEN, Shanghai, China) after being digested with *Nde*I and *Xho*I (TAKARA Biotechnology Co., Dalian, China) to create a recombinant pET21a–CAST.

Expression of the His-tagged CAST protein and analysis of its solubility

E.coli BL21 (DE3) transformed with pET21a-CAST was cultured in Luria–Bertani (LB) medium containing 100 μg/ml ampicillin, and grown at 37°C in a flat rotating incubator until the optical density (OD_{600nm}) of the culture reached 0.6. Expression of the fusion protein was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM for 4 h. Total bacteria were harvested after a centrifugation step at 10,000×g for 10 min. Pellets from 300 ml culture were re-suspended in 10 ml lysis buffer (pH 8.0) supplemented with 50 mM sodium phosphate, 500 mM NaCl and 1.0 mM phenylmethyl sulfonylfluoride (PMSF). The suspension was then subjected to ultrasonication (10 s with 20 s pauses at 300 W) on ice until clear. The translucent sonicates were then centrifuged at 12,000×g for 30 min at 4°C.

Two fractions of bacterial proteins were obtained, the soluble part (supernatant) and the insoluble part (cellular debris). The solubility of the His-tagged CAST protein was analysed by 8% SDS-PAGE.

Purification of the recombinant protein

The soluble fraction from cell lysate was kept in a water bath for 15 min at 90°C. Following the heat treatment was a centrifugation step at 12,000 ×g for 30 min and the supernatant was used for purification of the His-tagged CAST protein. The recombinant protein was purified using Ni-NTA resin (OIAGEN, Beijing, China) following the manufacturer's protocol. Briefly, a gravity-flow column packed with Ni^{2+}-NTA agarose was equilibrated with the binding buffer (pH 8.0, containing 20 mM sodium phosphate, 500 mM NaCl and 5 mM imidazole). Then the supernatant containing the His-tagged protein was loaded on it with 1 ml Ni-NTA matrix binding about 10-15 ml supernatant. The column was then washed successively by ten bed volumes (BV) of binding buffer and wash buffer for sufficient removal of contaminants. The wash buffer was different from the binding buffer only in the concentration of imidazole − 25 mM instead of 5 mM. Afterwards, the target protein was eluted by elution buffer and the eluates were collected for SDS-PAGE analysis. The elution buffer was supplemented with the same components as the binding and wash buffers except that the concentration of imidazole was increased to 250 mM. Eluates containing the fusion protein were dialysed against a dialysis buffer at 4°C (20 mM sodium phosphate, 100 mM NaCl, 1 mM DTT, 1 mM PMSF and 10% glycerol at pH 7.5). After dialysis the protein solution was stored at -80°C for subsequent use.

Production of polyclonal antibody against the recombinant CAST protein

The purified His-tagged CAST protein was dissolved in 0.01M phosphate buffered saline (PBS, pH 7.5) with a final concentration of 1 mg/ml and used for preparing antibodies in rabbits. Pre-immune serum was collected prior to immunization. Three-month old Japan White rabbits weighing 1.75-2.25 kg were initially immunized with 0.5 mg purified His-tagged CAST protein in an equal volume of complete Freund's adjuvant (SIGMA, Shanghai, China) by intramuscular injection (more than 5 sites per animal). Two booster injections were given with the same amount of His-tagged CAST protein mixed with an equal volume of incomplete Freund's adjuvant at a two-week interval. Two weeks after the last boost, the antiserum was harvested from the carotid artery and stored at -20°C.

Determination of the titre of anti-serum by ELISA

ELISA was performed to determine the titer of the polyclonal antibody raised against chicken CAST protein. Plates were coated with 0.4 μg purified His-tagged CAST protein per well. The polyclonal antibodies collected from rabbit were used as primary sera and titrations were performed with 25-, 50-, 100-, 200-, and 400-fold serum dilutions. With a primary antibody raised in rabbit horseradish peroxidase

(HRP) conjugated goat anti-rabbit IgG was used as the secondary antibody and OPD as the substrate for HRP. Pre-immune serum was used for the negative control. Individual serum samples were tested in duplicate and the two absorbance values at OD_{450nm} were averaged. It was considered to be a positive result when the ratio of test antibody and negative control exceeded 2.1.

Sequence alignment

Multiple sequence alignment of CAST proteins from different species was performed using Clustal X 2.0 programme and displayed using Genedoc software (http://www.nrbsc.org/gfx/genedoc).

Results and discussion

Construction of recombinant prokaryotic expression plasmid

The full-length chicken *CAST* gene coding sequence, which was composed of 2, 304 bp (without the stop codon) was sub-cloned successfully into the plasmid with *Nde*I and *Xho*I sites (Fig. 1). The accuracy of base pairs in the recombinant pET21a-CAST was confirmed by sequencing. The inserted fragment occurred identical with the cDNA sequence of chicken *CAST* gene on GenBank (GI: 212549669).

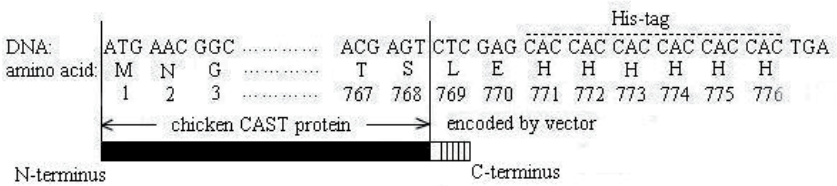

Fig.1-1. Structure diagram of the fusion His-tagged chicken CAST protein.

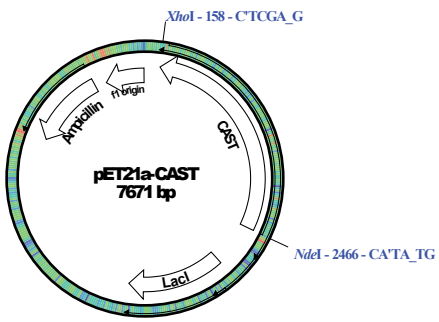

Fig.1-2. Full length chicken *CAST* ORF inserted into pET21a (+) vector.

Expression and purification of the His-tagged chicken CAST protein

Expression parameters including IPTG concentration (0.1, 0.5, 1.0 and 1.5 mM) and duration of induction phase (3, 4 and 5 h) were optimized to increase the yield of the C-terminal His-tagged CAST protein (data not shown). *E. coli* BL21 (DE3) transformed with pET21a-CAST exhibited a higher level of expression after induction with 0.5 mM IPTG for 4 h at 37°C (Fig. 2, lane 3 and lane 6). A distinct band of approximately 110 kDa was found only after induction, and no expression of this protein in BL21 (DE3) harboring pET21a-CAST was detected before IPTG induction (Fig. 2, lane 1 and lane 4.). The induced protein predominantly existed in the soluble fraction instead of the cell debris pellet, which suggested that the His-tagged CAST protein was soluble.

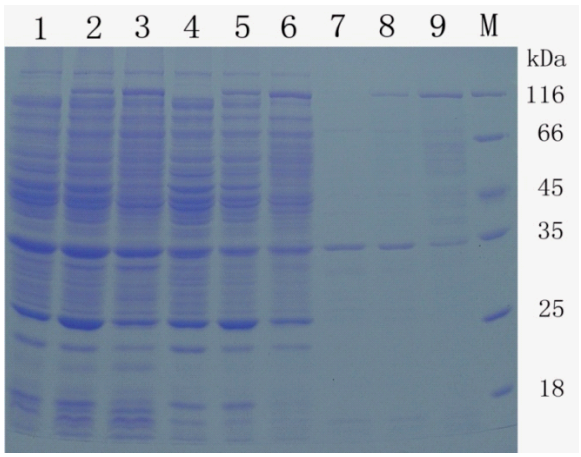

Fig. 2. SDS-PAGE analysis of the recombinant BL21/pET21a-CAST protein expression profile.
Lane M − protein marker; lane 1 − total cell lysate from BL21 (DE3) with pET21a-CAST before IPTG induction; lane 2 - total cell lysate induced at 0.1 mM IPTG; lane 3 − total cell lysate induced at 0.5 mM IPTG; lane 4 − soluble fraction of the cell lysate before IPTG induction; lane 5 − soluble fraction of the cell lysate induced at 0.1 mM IPTG; lane 6 − soluble fraction of the cell lysate induced at 0.5 mM IPTG; lane 7 − soluble fraction of the cell lysate after heat treatment before IPTG induction; lane 8 and 9 − soluble fraction of the cell lysate after heat treatment induced at 0.1 and 0.5 mM IPTG, respectively.

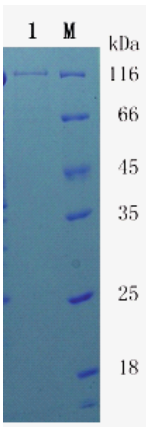

Fig. 3. SDS-PAGE analysis of His-tagged CAST after purification.

Human CAST protein was previously reported to be a heat-stable protein [Molinari and Carafoli 1997]. In the present study, recombinant protein exhibited the same property. We took the advantage of the heat stability of chicken CAST and used heat treatment as a very efficient step in the purification. Most proteins were removed by this denaturation step and the heat-stable fusion protein was still in the soluble fraction (Fig. 2, lane 8 and lane 9).

Purification of the His-tagged protein was performed with Ni-NTA resin column and a clear band corresponding to the molecular weight of about 110 kDa was revealed after the SDS-PAGE analysis without contamination of other bacterial proteins (Fig. 3).

The ORF of the fused protein encoded 776 amino acids, which including 768 amino acid residues of chicken CAST protein (NP_001131122.1) and a 6×His-tag at the C-terminal with Leu and Glu residues upstream the tag encoded by the vector. The theoretical isoelectric point was 5.65 and the predicted molecular weight was 82.51 kDa, *i.e*, slightly lower than the apparent molecular weight determined by SDS-PAGE (about 110 kDa). The discrepancy between the deduced molecular weight based on amino acid sequence and apparent molecular weight judged by relative mobility on SDS gels was reported in a study regarding a His-tagged fusion protein [Niu and Guiltinan 1994]. We assumed it might be caused by the strong basic charge of the recombinant protein as Lys residues ranked the highest percentage (13.5%) of the 20 constitutive amino acids. The six consecutive His residues at the C-terminal might also retard the mobility of fusion protein [Tang *et al.* 2000].

In this study, the fusion protein reached the highest expression at 37°C with 0.5 mM IPTG for 4 h. It was expressed in *E. coli* at a relatively low level and predominantly in the soluble form, which made it unprotected by the intracellular protease. As a consequence, trace amount of smaller bands, which represented the degradation of the fusion protein, could be detected by SDS-PAGE analysis during the whole purification procedure. In a study by Yasuhiko *et al*. [2000], the degradation of the fusion human CAST protein expressed in a prokaryotic expression vector pEX-2 was also revealed by immunoblotting assay. These results indicated that CAST was very sensitive to cytosolic proteases. In order to prevent proteases from digesting fusion protein after cell lysis, the addition of PMSF, the protease inhibitor, at a relatively high level of 1.0 mM was critical for the efficient purification of CAST protein.

Characterization of the polyclonal antibody against His-tagged CAST protein

ELISA was performed to evaluate the reactivity and specificity of the polyclonal antibody raised against the recombinant chicken CAST protein. Rabbit polyclonal antisera were able to recognize the purified His-tagged CAST protein specifically without cross-reaction with proteins from the lysates of BL21 (DE3) harbouring pET21a-CAST before induction. The titre of the polyclonal antibody was 1:200. The purified antigen along with its specific antibody, could further be used for isolation of immunoreactive molecules in tissue suspensions.

Multiple sequence alignment

The multiple sequence alignments of the full-length CAST proteins from different species were performed to identify the conservative domains. In domain organization, CAST molecules from 13 species consist of four typical repetitive domains (http://pfam.sanger.ac.uk/family/PF00748). Each domain contains about 140 amino acid

residues and has 20-30% sequence identity with each other. A consensus sequence of a seven-peptide (T-I-P-P-X-Y-R) exists in each repeating unit (Fig. 4).

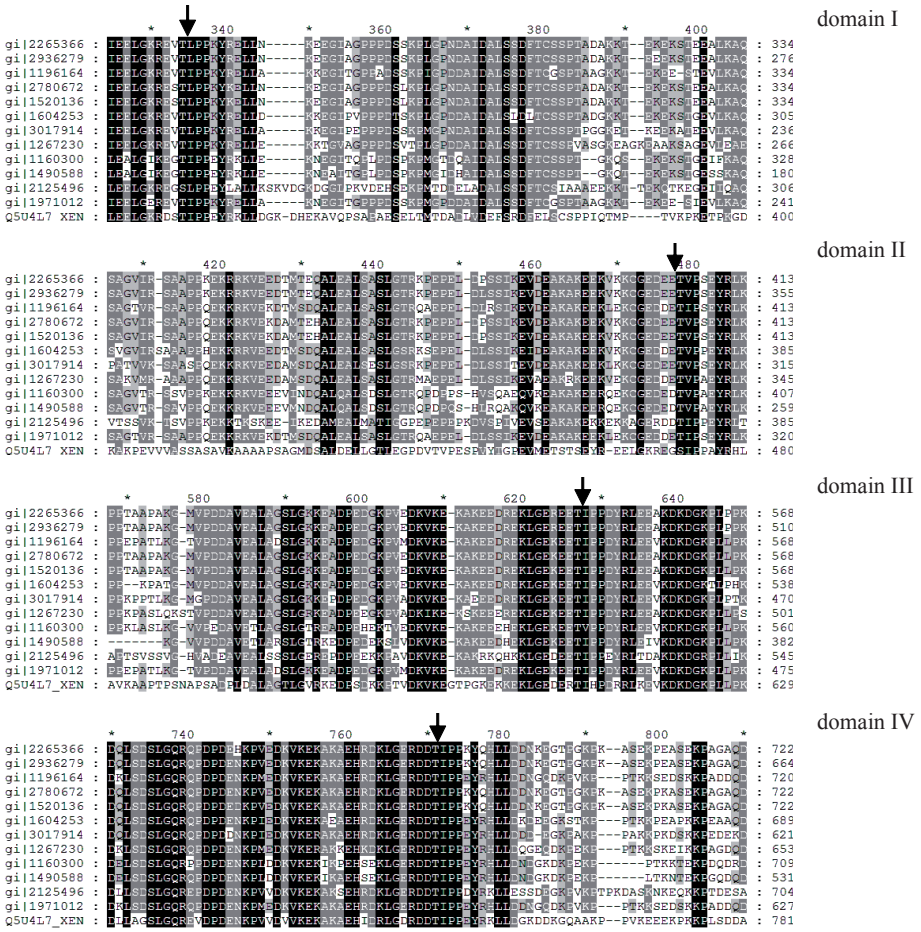

Fig. 4. Alignment of amino acid sequences of CAST from 13 species: gi|226536690|, sheep; gi|293627945|, goat; gi|119616478|, human; gi|27806721|, bovine; gi|152013655|, wild yak; gi|160425377|, pig; gi|301791454|, panda; gi|126723094|, rabbit; gi|11603006|, mouse; gi|149058897|, rat; gi|212549669|, chicken (deduced from the inserted chicken CAST mRNA sequence and identical with the NP_ 001131122.1); gi|197101221|, sumatran orangutan; Q5U4L7_XENLA, African *Xenopus laevis*. Conserved residues in each domain were marked by an arrow at the top of the alignment.

When amino acid composition was analysed, the chicken CAST protein was rich in hydrophobic residues and very poor in aromatic residues (accounting for 34.1% and 1.6% of the total 768 amino acids, respectively), which was a consistent feature of CAST proteins. Prediction of phosphorylation sites by NetPhos 2.0 (http://www.

cbs.dtu.dk/services/NetPhos) revealed 59 potential sites (1, 15 and 43 for Tyr, Thr and Ser residues, respectively) with an output score higher than 0.800. This indicated that chicken CAST might be the substrate for different protein kinases and modulate its activity through phosphorylation/dephosphorylation cycles.

Up till now, four forms of chicken CAST have been reported: NP_001131122.1 (GI: 212549669), ABP6838.1 (GI: 145411437), ENSGALP00000023613 and ENSGALP00000036895 with different length of 768, 747, 686 and 475 amino acid residues, respectively. They are produced by alternative splicing of different exons in the N-terminal region of the transcript as reported in other species [Li and Goldberg 2000, Takano *et al.* 2000]. All four forms have 4 repetitive units with each containing the conservative sequence (T-I-P-P-X-Y-R), which was considered to be the binding site of calpain [Emori *et al.* 1987]. The transcriptional changes in the N-terminal region of a CAST molecule might be responsible for determining its cell localization, modulating its specificity and calpain inhibitory efficiency [Averna *et al.* 2003]. Further studies focused on recombinant chicken CAST with various deletions in the N-terminal region will help to get insight the molecular mechanisms underlying the exon skipping or alternate splicing.

In the present study, we have expressed and purified the C-terminal His-tagged chicken CAST protein as a full length molecule and obtained the specific rabbit polyclonal antibody against the recombinant protein. The establishment of ELISA procedure for quantification of CAST protein will help to identify factors that might lead to the variations of its concentration in muscle and better understand the mechanism underlying the process of meat tenderization. Our result also provides a good tool for further investigating the binding characteristics of CAST regarding its affinity and specificity for different ligands, studying its concentration changes in response to the variation of muscle Ca^{2+} content and indentifying cellular interaction partners by coimmunoprecipitation as well.

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