

MONITORING THE DEGREE OF THE FROZEN DENATURATION OF SKELETAL MUSCLE MYOSIN BY ELISA METHOD

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SUMMARY

The degree of denaturation of bovine myosin during the freezing period was determined by indirect ELISA method. Antiserum was produced from rabbits immunized with bovine myosin. Cross reactivity of anti-bovine myosin IgG to porcine, rabbit, and chicken myosin was 16.2, 4.0, and 2.7% to bovine myosin. The rate of myosin denaturation were observed by the condition of the storage at -20°C , and the repeated freezing and thawing process and compared with imported frozen meat in Korean market. Solubility of bovine muscle proteins slightly increased until 80 days, but the reaction of antibody to myosin in the solution started to decrease at 50 days ($p < 0.05$). During the repeated process of freezing and thawing with 10 day interval, anti-myosin reaction was decreased about 50% after the second thawing, and decreased below 25% after the fifth ($p < 0.05$). The myosin denaturation during the repeated process of freezing and thawing happened more severely than that during the normal storage process. The frozen denaturation of myosin might be due to the denaturation of myofibrillar proteins during freezing period, and result in reducing myosin epitope.

INTRODUCTION

During freezing process, the decrease of protein solubility, denaturation of myofibrillar proteins (mainly myosin), change of color, and thickness of structure by drip loss damage the quality of meat (Wagner et al., 1985; 1986 I,II; Dobraszzyk et al., 1987; Lannari et al., 1991). Therefore, the price of frozen meat and chilled meat has been differentiated, and frozen meat have been often sold as chilled meat after thawing illegally. Gottesmann and Hamm (1983) had experimented the assay of β -hydroxyacryl-CoA-dehydrogenase (HADH) by both spectrometric and colour test, and Chen et al (1988) had worked distinguishing between fresh meat and frozen-thawing meat by APIZYM system applying difference the release rate of Mitochondrial Enzymes. In recent the researches to immunoassay have been actively advanced, and have been applied in the field of food science, especially identification and measurement of denaturation of structure proteins (Martin et al., 1991; Sargeant 1993; Rodriguez et al., 1991). In the present experiment we have produced polyclonal antibodies to native bovine myosin, antigen, isolated and purified IgG, and researched the degree of denaturation of degenerated or destroyed myosin through freezing process.

MATERIALS & METHODS

Antibody production and purification

Polyclonal antibodies against bovine myosin (Antigen; Ag, Sigma, USA) were raised in New Zealand White male rabbits injecting Ag solution (0.01M phosphate buffered saline pH 7.4, 0.15M NaCl) with same volume of Freund's Complete adjuvant (Ag 100 μg in the solution 1ml). After 2 weeks the same solution with Freund's Incomplete adjuvant was injected. After 5 times injection, the blood was collected using heartpuncture. Serum was separated and IgG was purified through Affinity-Gel protein-A agarose column. IgG solution (0.01M PBS pH 7.4) was compensated to 4mg/ml, added to the same content of glycerol, and stored at -20°C .

Indirect ELISA method

Competitive indirect method was appropriated as the coating type of not established Ab but Ag. One percent gelatin solution was made up of blocking solution, and titration of Ab was tested. The secondary Ab was used Goat anti-rabbit IgG conjugating H.R.P. (Horse radish peroxidase). Washing solution was PBS buffer with tween 20 (0.05%), and reaction time was 2 hours at 37°C. substrate was OPD (O-phenylenediamine dihydrochloride) solution, and stop solution was 2.5N H₂SO₄. O.D. values measured at 490nm. The specificity of Ab between species was tested by Shin et al method (1993). Cross reactivity was individually investigated through compared porcine, rabbit, and chicken myosin with bovine myosin, standard. In order to obtain precision through evaluating the reproducibility of this assay system. Intra-inter assay variation was also tested by Kim method (1983).

Sample treatment

Passed 24 hours after slaughter, beef *M. semitendinosus* of 10 individuals was obtained. Samples for freezing period test were prepared, removed visible adipose and connective tissue, cutted 1cm thick, vacuum packaged, and stored -20°C. Thawed every 10 day samples were used to experiment. For observing the degree of denaturation of Ag during repeated freezing-thawing process, the same parts of carcass were obtained and prepared as the same methods, but only different cutting method, 5*10*8cm³ cubes (about 400g). Samples were thawed at 5°C with 10 day interval, and refrozen after sampling. The comparison of fresh meat with imported frozen meat distributed in Korean market was carried out with randomly obtained imported frozen beef *M. semitendinosus* and fresh meat passed 24 hours after slaughter. Protein concentration in the equally buffered saline solution (0.5M KCl, 10mM potassium phosphate buffer pH 6.8 at 5°C; BSS) was measured by biuret method, and Ag content in the solution was detected by ELISA method. Control 2g was obtained at chilled meat before freezing treatment, added to 18ml BBS, homogenizing for 3 mins, and centrifuged at 10,000rpm for 30mins at 4°C. Supernatant was filtered through Whatman No. 3 and filtrates were used at the experiments. Standard protein solution was bovine serum albumin (10mg/ml). During freezing period, repeated thawing-freezing and the comparison of fresh meat with imported frozen meat were executed by the same procedure.

RESULTS & DISCUSSION

The formation of anti-serum to myosin was certificated by indirect ELISA method and titration of Ab was 1:16,000. The isolation and purification of IgG applied into AFFi-Gel protein-A Maps I Kit improving general protein-A agarose method showed higher recovery (3mg/ml) than established method. At Ag coating concentration: 10µg/ml, primary Ab dilution multiple: 1:5000, and secondary dilution multiple: 1:20,000, B1/B0 value appeared 50%. Therefore, the following experiments were accomplished under this condition. Making standard curve appeared into Figure 1. Detection range was approximately from 0.5µg/ml to 500µg/ml. Sensitivity was about 0.5µg/ml, this sensitivity was slightly lower than that of Dincer et al (1987) report (0.1µg/ml), but this was out of question in the part of measurement of denaturation. The cross reactivity of the various Ags was showed at Table 1. The cross reactivity of the various Ags was showed at Table 1. This result shows epitopes have the differences belong to species. Intra-inter assay variance value was Table 2. Recovery test for the investigation of propriety appears on Table 3.

Protein solubility during freezing period showed chilled condition (0 day) was 2.14mg/ml ($p < 0.05$), and increased to 2.95mg/ml until 80 days (Fig. 2) ($p < 0.05$). These results presented the same inclination of results obtained by Wagner et al (1986). They reported solubility weakly increased until about 13 weeks and decreased during storage at -20°C. Figure 3. showed the decline of solubility in the experiment of repeated freezing-thawing treatment. Solubility started to decrease at the third thawing. This result showed the decrease of myofibrillar proteins is due to repeated freezing-thawing. Myosin concentration increased until 50 days with solubility relatively, but from next step decreased. This may be due to denaturation and destruction of myofibrillar proteins (mainly myosin), and structural change of epitopes related to Ag-Ab reaction. Detected myosin concentration rarely changed at the first thawing, but from the second it severely declined, and was 0.5µg/ml ($p < 0.05$) to the fifth. This result appears that the reaction of Ab to myosin extensively decrease relatively, even though solubility also decrease. Figure 4. showed the result of the comparison of fresh meat with imported frozen meat distributed in Korean market. Imported frozen meat may be considered through 2nd

- 3th thawing treatment. Comparing the amount of myosin to solubility, the result showed the range was 50 - 75%, the difference of each sample existed.

CONCLUSION

At our results, we can easily grasp the degree of denaturation of myosin during freezing period. The denaturation of myosin appeared after 50 days during freezing period. Repeated thawing-freezing process, the determination appeared easily from the second thawed sample. The degree of denaturation at imported frozen meat showed the range of 50-75% myosin content in comparison to that of fresh meat. In case of thawing imported frozen beef in very long frozen storage (over 3 months) may be able to distinguish between freezing-thawing meat and fresh meat by ELISA method.

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