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ELISA Based Quantification of Chicken Specific Troponin-T Peptide in Skeletal Muscle TCA Extracts

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Introduction

Skeletal muscle troponin consists of the TnC (the sensor), TnI (the regulator), and TnT (the link to the muscle thin filament). Troponin T (TnT) represents the tropomyosin (Tm)-binding subunit of the troponin (Tn) complex whose role resides in the Ca^{2+}-dependent regulation of vertebrate striated muscle contraction. TnT interacts with its other counterparts and may be considered as an organizer molecule [1]. Moreover, due to the complex splicing of the NH_2-terminal variable region, a large number of fast skeletal muscle TnT isoforms are expressed in contrast to cardiac and slow skeletal muscle TnTs, and all of them are differentially expressed during myogenesis and development in a tissue specific manner [2]. In postmortem muscle, after rigor mortis, actin and myosin do not present enhanced degradation [3]. Several key myofibrillar proteins, however, have been shown to be degraded at differing rates [4]. TnT degradation and the detection of fragmentation products, represent a widely reported and well-established marker of skeletal muscle aging also in several animal products intended for commercial distribution in the food industry [4-7]. TnT fragments have been detected in various animal species such as beef, rabbit, lamb, fish, camel and poultry and have been positively associated with increased meat tenderness during aging [8-13]. The effect of aging on the chicken meat quality has been reported in a study by Wei *et al*., who observed in muscle extracts a significant increase in two specific peptides, which were later identified as products of chicken fast skeletal muscle TnT degradation [8].

Based on our previously developed competitive ELISA for the detection and quantification of TnT (16-31) fragment in trichloracetic acid (TCA) soluble beef skeletal muscle extracts [6, 14], as a marker of meat tenderness, an ELISA was similarly/respectively developed for the detection of the 21 aa TnT fragment EPAPPPEEKPRIKLTAPKIPE, which was reported to be present, in chicken skeletal muscle extracts [8].

Results and Discussion

In silico analysis of the reported peptide sequence in NCBI database led to the identification of the 21aa fragment as part of the fast skeletal muscle TnT isoforms (*TNNT3* gene) present in *Gallus gallus* and conserved in many other bird species. The fragment spans from aa position 61 to 81 of the 287 total aa protein (NP_990253.1) (Figure 1). This fragment will further be referred as TnT-21.

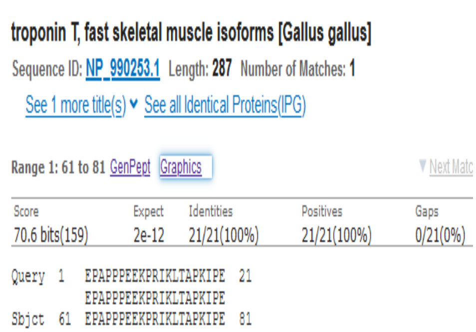

Fig. 1. In silico analysis of EPAPPPEEKPRIKLTAPKIPE peptide.

High affinity polyclonal anti-TnT-21 antibodies were generated in New Zealand white rabbits through a 3-dose immunization protocol with KLH as carrier, according to established methods. Immunization led to highly reactive antisera against TnT-21 (Figure 2a).

Isolation and purification of specific anti-TnT antibodies through protein A column (1st step) and TnT-

21 immunoadsorbent (2nd step) resulted in purification of highly specific anti-TnT-21 polyclonal antibodies as confirmed by indirect ELISA against TnT-21 (Figure 2b).

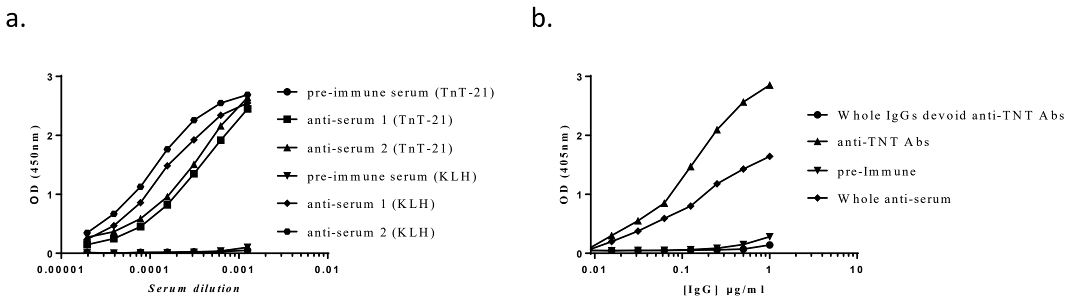

Fig. 2. a. Indirect ELISA against TnT-21 (2μg/ml) and KLH (1μg/ml) immobilized on the microplates, for the evaluation of rabbit immunization effectiveness. Antiserum 1 & 2 correspond to the samples after two consecutive booster immunizations in-between 1-month period. b. Indirect ELISA against TnT-21 (2μg/ml) for the evaluation of anti-TnT-21 Abs purification and reactivity.

To develop the competitive ELISA procedure, affinity-purified anti-TnT-21 Abs were conjugated to biotin using glutar-aldehyde, and then commercial peroxidase-labelled streptavidin was used as detection system. The ELISA is based on the binding inhibition of anti-TnT-21 Abs to immobilized TnT-21 by soluble TnT-21. For the measurement of chicken TnT degradation in muscle tissue and potential delivery of the respective TnT-21 peptide as catabolic product, the conjugate anti-TnT-21 affinity purified Ab-biotin was co-incubated (v/v) with soluble peptide at serial dilutions for the standard curve formation. After optimization, biotin labelled anti-TnT-21 Abs were co-incubated at a final concentration of 200ng/ml and the respective standard curve had useful range 15pmol to 500pmol TnT-21/well (100μl/well). The results were expressed as inhibition percent (%) plotted versus the concentration of inhibitor TnT fragment in pmol/well (Figure 3).

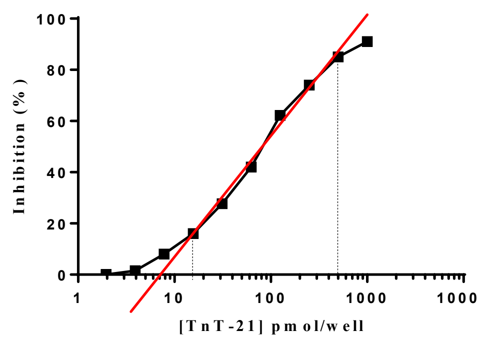

Fig. 3. Standard curve of competitive ELISA. The working volume per well is 100μl. The useful range for TNT quantitation is 15pmol to 500pmol TnT-21/well.

For TnT-fragments tissue analysis, thirty (30) TCA muscle extracts were produced based on previously described methods [7] from conventional chickens (C), free range chickens (FR) and FR chickens in which aromatic herbal extracts had been added to their diet (FRp) (Table 1). All chickens were raised under industrial conditions for commercial food consumption. Chicken thigh muscles were excised at slaughter, just before product processing, and muscle samples were immediately stored at -80°C until use. For the aging procedure thigh muscle pieces, prior to the experiment, were vacuum packed in plastic sealed bags, after removing visible fat and connective tissues and afterwards thawed at 4°C for 0, 24, 72 hours and 7 days [8-13]. Measurement results for different time points of *post mortem* aging are shown at Figure 4.

Table 1. Chicken[*]/ tissue specifications.

Genotype	*Type*	*Raise Conditions/ Diet*	*Skeletal muscle tissue*	
			N	*Weight/pc (mean ±SD)*
Ross 508	Fast Growth	Conventional (C)	10	7±2.3 gr
Sasso	Slow Growth	Free range (FR)	10	6±3.1 gr
		Free range + Aromatic herbals (FRp)	10	6±4.4 gr
Total			30	19±3.7 gr

[*]*all chickens were raised under industrial scale production for food/meat consumption*

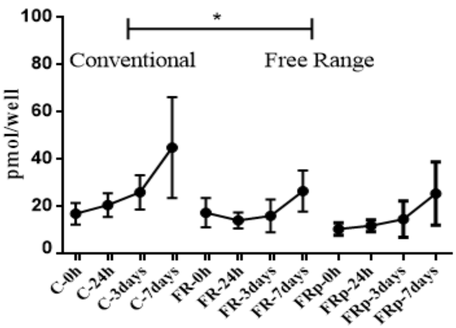

Fig. 4. Measurements of soluble TnT-21 in chicken's skeletal muscle 5%TCA extracts. Post-mortem aging process of skeletal muscle tissue was held at 4°C in 4 distinct time points (0, 24, 72 and 168 hours).

A Troponin-T fragment, a protein degradation product, was detected in chicken skeletal muscle TCA extracts and was identified as a potential marker of *post mortem* aging. Our quantitative competitive ELISA for specific TnT-21 fragment in free range poultry will be further used for the correlation analysis between TnT-21 concentration and qualitative characteristics of the meat during *post mortem* aging. This quantitative ELISA may be proved advantageous for future use at the research and industrial level.

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References

1. Vinogradova, M.V., Stone, D.B., Malanina, G.G., Karatzaferi, C., Cooke, R., Mendelson, R.A., et al. *Proc Natl Acad Sci*. **102**, 5038-5043 (2005), https://doi.org/10.1073/pnas.0408882102
2. Jozaki, M., Hosoda, K., Miyazaki, J.I. *J Muscle Res Cell Motil*. **23**, 235-243 (2002), https://doi.org/10.1023/a:1020956216423
3. Li, S., Xu, X., Zhou, G. *Poult Sci*. **91**, 150-160 (2012), https://doi.org/10.3382/ps.2011-01484
4. Iwasaki, T., Taniguchi, H., Hasegawa, Y., Maeda, N., Yamamoto, K. *J Sci Food Agric*. **96**, 3944-3949 (2016), https://doi.org/10.1002/jsfa.7558

5. Huang, M., Huang, F., Xu, X., Zhou, G. *Food Chem*. **115**, 181-186 (2009), https://doi.org/10.1016/j.foodchem.2008.11.095
6. Zhang, Y., Zhang, D., Huang, Y., Chen, L., Bao, P., Fang, H., et al. *Food Chem*. **318**, 126516 (2020), https://doi.org/10.1016/j.foodchem.2020.126516
7. Tsitsilonis, O.E., Stoeva, S., Echner, H., Balafas, A., Margomenou, L., Katsoulas, H.L., et al. *J. Immunol. Methods* **268**, 141-148 (2002), https://doi.org/10.1016/S0022-1759(02)00186-2
8. Wei, Z., Muraoka, J., Tanabe, S., Nishimura, T. Changes in taste components of japanese native fowl hybrid during postmortem conditioning. 48"' ICoMST-Rome, 25-30 August 2002 - Vol.1.
9. Zhao, L., Xing, T., Huang, J., Qiao, Y., Chen, Y., Huang, M. *Anim. Sci. J*. **89**, 423-431 (2018), https://doi.org/10.1111/asj.12921
10. Wang, J., Zhou, J., Chen, Y., Zhang, X., Jin, Y., Cui, X., et al. *J. Anim. Sci. Biotechnol.* **10**, 1-10 (2019), https://doi.org/10.1186/s40104-019-0389-7
11. Zhang, X., Gao, T., Song, L., Zhang, L., Jiang, Y., Li, J.L., et al. *Int J Food Sci Technol*. **52**, 2097-2105 (2017), https://doi.org/10.5713/ajas.16.0435
12. Husak, R.L., Sebranek, J. G., Bregendahl, K. *Poult Sci*. **87**, 2367-2376 (2008), https://doi.org/10.3382/ps.2007-00294
13. Lin, C.-Y., Kuo, H.-Y., Wan, T.-C. *Asian-Australasian J Anim Sci.* **27**, 880 (2014), https://doi.org/10.5713/ajas.2013.13646
14. Voelter, W., et al. *J. Prakt. Chem*. **342**, 179-191 (2000), https://doi.org/10.1002/(SICI)1521-3897(200002)342:2<179::AID-PRAC179>3.0.CO;2-2