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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation

Structural and functional analyses of egg white protein ovalbumin-related protein X in chicken embryo
（鶏卵白タンパク質ovalbumin-related protein Xの胚発生における構造と機能の解析）

学位論文要約：
Dissertation Summary

Egg white, seen in avian and reptile eggs, is located in the space between egg yolk and egg shell. Physiological roles of egg white in embryo development have been well-documented in chicken egg. Egg white provides nutrition for developing embryo and protects egg yolk against physical shock and microbial infections. Protein is a key component to the above-mentioned chemical and physical properties of egg white (nutrition supply, physical shock absorption, and antimicrobial action). There exist 148 kinds of proteins in chicken egg white. Egg white proteins play a role in serving amino acids for development of chick embryo. Some egg white proteins, ovotransferrin (OTF) and lysozyme (LYZ), exert antimicrobial action to protect egg yolk harboring an embryo from microbial infection. In addition to the roles in assisting the growth of embryo, more direct involvement in embryo development has been proposed for ovalbumin (OVA). It has been reported that small amount of OVA existing in egg white is incorporated in its intact form into the chick embryo. This fact suggests that OVA may have more direct functions in developing embryo, in addition to contributing as the storage protein to serve amino acids into embryo. Besides OVA, some other proteins, such as OTF and LYZ, were detected in embryonic organs and/or blood system. However, there is yet little information on these proteins' functions involved in embryo development.

In major egg white proteins such as OVA, OTF, and LYZ, numerous studies have been conducted to characterize physicochemical and structural properties of these molecules. On the other hand, few studied have focused on minor egg white protein due to the difficulty of purification. Ovalbumin-related protein X (OVAX) is one of the minor egg white protein and its functions remain unclear. OVAX is a glycoprotein with molecular weight 45430 Da and pI 6.29. Recently, OVAX was reported to interact with heparin, a negatively charged glycosaminoglycan. Heparin and its analog heparan sulfate (HS) play crucial roles in multiple biological processes, such as cell proliferation, cell migration, and cell differentiation during embryogenesis. This raises the possibility that the heparin-binding protein OVAX participates in embryo development through binding to heparin and HS.

The aim of this study is to figure out OVAX's biological functions during embryo development. The first study was to investigate whether OVAX migrates from egg white into embryo and whether OVAX is localized in embryonic organs. The second study was to estimate heparin-binding affinity of OVAX in embryonic stages by monitoring the change of heparin-binding affinity of the protein in egg white during incubation of egg at 37℃. The third study was to investigate the participation of OVAX in chondrogenic activity of a growth factor bone morphogenetic protein 2 (BMP2) as a model system for the exploration of OVAX's functions in embryo development.

Firstly, whether egg white OVAX migrates into chick embryo was investigated by immunological methods. No cross-reacted protein was detected in egg yolk of egg before incubation (0d-egg); while a 50kDa protein band

was observed in egg yolk of egg incubated at 37 ℃ for 10 days (10d-egg) and egg incubated under the same conditions for 16 days (16d-egg). The 50kDa protein band was thicker in 16d-egg yolk than in 10d-egg yolk. The increase in OVAX level during incubation implies that OVAX, although it does not exist in yolk in early incubation stage (0d-egg), accumulates in yolk in middle and later stages (10d- and 16d-egg). Thus, OVAX was localized in the yolk of egg incubated for 10 days or longer. These results demonstrate that egg white OVAX moves into yolk during the embryo development. Immunohistochemical analysis of embryo resided in 10day-incubated eggs showed that OVAX was detected in most of the organs, including brain, eye, heart, liver and stomach. These suggest that OVAX is incorporated from egg white into the embryo through egg yolk.

Secondly, heparin-binding affinity of OVAX in egg white of fertilized egg was assessed. Each of egg white separated from 0d-egg and 10d-egg was subjected to heparin sepharose chromatography. Elution profile of egg white protein separated from 0d-egg showed that a broad shoulder appeared in the NaCl concentration range of 0.10 – 0.23 mM, donated as P-1, and three discrete peaks were observed in the concentration range of 0.24 – 0.39 mM, 0.40 – 0.49 mM, and 0.50 – 0.60 mM which is denoted as P-2, P-3, and P-4, respectively. The elution profile of 10d-egg showed that four discrete peaks which were observed at the same salt elution positions as P-1 to P-4 of the 0d-egg's profile. As compared with the 0d-egg's profile, the peak area of P-4 peak was diminished in the 10d-egg's prolife. On the contrary, the three peaks of P-1, P-2, and P-3 were greatly expanded in the 10d-egg's profile. The abundance ratio of OVAX in each peak was determined by ELISA. In 0d-egg, OVAX was mainly eluted at P-3 and P-4, of which their abundance ratios were 55.7±2.3% and 33.3±3.1%, respectively. In 10d-egg, OVAX eluted at P-4 was a minor protein as indicated by its small amount (9.6±0.4%). Alternatively, OVAX eluted at P-2 was dominant, accounting for 61.5±3.0%. These results indicate that elution of OVAX partially shifted from P-3 and P-4 to P-2 after incubation for 10 days.

Here, OVAX eluted at P-1, P-2, P-3 and P-4 were denoted as $OVAX_1$, $OVAX_2$, $OVAX_3$ and $OVAX_4$, respectively. $OVAX_4$ residing mainly in 0d-egg and $OVAX_2$ residing dominantly in 10d-egg were used for a stoichiometric analysis of heparin-binding affinity. The stoichiometric analysis of the two OVAX molecular species was carried out by isothermal titrating calorimetry (ITC) using fondaparinux (a low molecular weight heparin) as a ligand. Dissociation constant value (K_d) of $OVAX_2$ was 0.185 μM, which is two times higher than that of $OVAX_4$ (0.096 μM). The difference in K_d value was subtle, but the data clearly support the results of heparin sepharose chromatography that $OVAX_2$ has weaker heparin-binding affinity than $OVAX_4$. In contrast, the number of fondaparinux bound to $OVAX_2$ was ca.3, which is nearly identical to the number for $OVAX_4$. In sum, ITC results showed that $OVAX_2$ has equal number of heparin-binding domain with $OVAX_4$, but has slightly weaker heparin affinity than $OVAX_4$ dose.

To elucidate why $OVAX_2$ has weaker heparin-binding affinity than $OVAX_4$ does, structural properties of $OVAX_4$ and $OVAX_2$ were investigated by measuring tryptophan fluorescence and circular dichroism (CD) spectra. The fluorescence and near-UV CD results suggest that tertiary structure of $OVAX_2$ is not significantly different from $OVAX_4$. The far-UV CD spectra showed that the $OVAX_2$ spectrum was highly similar to the $OVAX_4$ spectrum. However, secondary structure predicted from the far-UV CD spectra showed that $OVAX_2$ had 3.6% less α-helix and 1.9% more β-sheet than $OVAX_4$ does. These results suggest that the change in secondary structure of OVAX causes in decline in heparin-binding affinity of the protein. Protein surface charge of the two proteins was characterized by zeta potential measurement. The zeta potential of $OVAX_2$ at pH 7.0 was -8.79 mV which was significantly lower (P<0.05) than that of $OVAX_4$ (-5.17 mV); suggesting that $OVAX_2$ has more negative surface charge than $OVAX_4$. These results suggest that the change in secondary structure might be closely related to the acidification of protein surface charge. The transition from $OVAX_4$ to $OVAX_2$ that occurred during the incubation of fertilized egg was accompanied with acidification of OVAX surface charge, which might have resulted in weakening the heparin-binding affinity of OVAX.

Biochemical analyses of OVAX contained in egg white showed that there exist four OVAX types that differ in heparin-binding affinity (in descending order, $OVAX_4$, $OVAX_3$, $OVAX_2$, and $OVAX_1$). After incubation of egg for 10 days, $OVAX_2$ became the dominant OVAX in egg white. Thus, $OVAX_4$ is altered to a low heparin-affinity $OVAX_2$ via $OVAX_3$ during embryo development. The spontaneous transformation of OVAX might be concerned with the control of functions of heparin and HS during embryogenesis. Heparin and HS can interact with growth factors (e.g. bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and hedgehogs) which play significant roles in organogenesis of embryo. Especially in the HS, there are numerous reports on controlling the function of the growth factors in organogenesis. HS regulates organogenesis by controlling distribution, bio-availability and action on target cells of the growth factors during organogenesis. These facts suggest a possibility that OVAX could participate in organ development thorough binding to HS during embryogenesis.

One of the events regulated by HS during embryo development is chondrocyte differentiation. HS regulates chondrocyte differentiation through binding to BMP2 that is known to be a potent promoter of cartilage differentiation in embryo development. The participation of OVAX in the control of BMP2 activity during chondrogenesis was investigated using micromass culture system which supports chondrocyte differentiation of mesenchymal cells. In micromass culture, cartilage nodules that differentiated from mesenchymal cells can be stained with alcian blue. The amount of cartilage matrix was quantified by measuring the optical density (600 nm) of dye extracted from alcian bule stained culture. The intensity of micromass culture treated with 100 ng/ml BMP2 (BMP2-culture) was 3.0 times higher than that of control culture (micromass culture treated with neither BMP2 nor OVAX), clearly indicating that BMP2 induced chondrocyte differentiation. Micromass culture treated with BMP2 plus $OVAX_4$ showed higher intensity than BMP2-culture did. The intensities of cultures treated with 100 ng/ml BMP2 plus 10 μg/ml $OVAX_4$ and 100 ng/ml BMP2 plus 100 μg/ml $OVAX_4$ were 1.6 and 1.7 times higher than that of BMP2-culture. These results suggest that $OVAX_4$ enhanced the chondrogenic activity of BMP2. On the other hand, $OVAX_2$ did not affect the BMP2 activity. The BMP2-promoted chondrogenic differentiation is regulated by phosphorylation of smad, the transcriptional mediators of BMP signaling. Whether $OVAX_4$ affects BMP2-mediated phosphorylation of smad was examined by using immunostaining with an anti-phospho-smad1/smad5/smad9 antibody. Immunostaining of phospho-smads was more intense in the culture treated with BMP2 plus $OVAX_4$ than in BMP2-culture. These results demonstrate that $OVAX_4$ increases in the level of BMP2-mediated smad phosphorylation.

Analysis with micromass culture system showed that $OVAX_4$ enhanced the chondrogenic activity of BMP2 by increasing in the level of BMP2-mediated smad phosphorylation. On the other hand, $OVAX_2$ did not affect the BMP2 activity. Taken together with the fact that $OVAX_4$ was spontaneously altered to $OVAX_2$ during embryo development, it is suggested that the alternation in heparin-binding affinity of OVAX modulates the BMP2 activity during embryogenesis. This is the first report showing that egg white protein might be involved in organogenesis during embryonic development.