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Characterization and Distribution of Transferrin from the Last Larval Haemolymph of *Papilio xuthus*

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Transferrin is a molecule carrying iron to store and maintain for iron homeostasis of living organisms. In this study, we have purified transferrin, as an iron-binding protein, from the last larval haemolymph of *Papilio xuthus* by KBr density gradient ultracentrifugation and gel filtration (superose 6 HR) using fast protein liquid chromatography (FPLC) and transferrin containing iron was identified by Ferene S staining. The purified haemolymph transferrin was shown to have molecular mass of 78 and 80 kDa and amino acid composition of transferrin was rich in aspartic acid, valine, leucine and glutamic acid. With immuno-diffusion assay, we confirmed the existence of the transferrin in the haemolymph and fat body by detection of visible and clear positive reaction. From the quantitative comparison by rocket immuno-electrophoresis process, the amount of transferrin were increased in the haemolymph of 3 days after pupation and the whole 5 days after pupation. Here, with biochemical and immunohistochemical analysis, we speculate the relationship of transferrin between the physical characteristics and distribution during metamorphosis of *P. xuthus*.

Key words – *Papilio xuthus*, haemolymph, fat body, transferrin

Introduction

Iron is an essential nutrient and a potential toxin in all living organisms. It serves as a catalytic core during oxidative reaction, which in some instances results in destructive oxidative stress [14]. Transferrin is one of iron transport proteins and plays an important role in iron metabolism. Iron in aqueous solution has ready access to two stable oxidation states, the ferrous, Fe(II), and the ferric, Fe(III). This property implies the participation of iron in biochemical reactions, including those controlling the flow of electrons through bioenergetic pathways [11]. Transferrin and transferrin-like proteins are relative newcomers to the evolutionary scene, being found only in the phylum Chordata. Bartfeld and Law [2,3] subsequently characterized the low molecular weight protein as an insect transferrin. *Manduca sexta* transferrin is a glycosylated polypeptide of 77 kDa with a single iron-binding site and 29% amino acid sequence identity with human transferrin. A transferrin-like protein also has been identified from the haemolymph of a cockroach, *Blaberus discoidalis* [8].

The purpose of the this study is characterization and identification of insect transferrin. Transferrin is 74 kDa protein carrying an one Fe^{3+} ion in insects whereas two in other invertebrates [13]. Yet, little is known about characteristics of transferrin as well as iron transport and utilization in invertebrates, particularly insect [13]. Huebers *et al.* [7] demonstrated the participation of transferrin (Fe transport) in the delivery of iron ions from the haemolymph to the fat body and used by primarily muscle and epidermis in *Manduca sexta*. Especially, in insect, transferrin serves as the major extracellular iron transport protein [13]. Insect transferrin have been reported from five insect species. Whereas other insect transferrins from a moth (*Manduca sexta*), flesh fly (*Sarcophaga peregrina*), fruit fly (*Drosophila melanogaster*) and mosquito (*Aedes aegypti*) have one iron-binding site, cockroach (*Blaberus discoidalis*) transferrin has two iron-binding sites and strongly resembles vertebrate serum transferrins [12]. The properties of insect transferrins suggest that they may have similar functions to vertebrates. Insect transferrin was little investigated on the view of mechanism of oxidative stress as antioxidant. Moreover, the strict role and secretory pathway of transferrin were not revealed clearly. Therefore, limited information is available in characteristics and regional distribution even though it is expected distinct role of transferrin in iron metabolism exists due to the diversity of in-

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sect species. In this study, we investigated the characteristics and distribution of transferrin during metamorphosis of *Papilio xuthuts*.

Materials and Methods

Animals

Papilio xuthus larvae were collected at the area of eastern Kum-Ho river, Daegu. Larvae were reared on *Poncirus trifoliata* leaves at room temperature. According to the interval of last instar larval (5L), prepupal (PP), just after pupation (P0), 1 day after pupation (P1), 3 days after pupation (P3), 5 days after pupation (P5), and 7 days after pupation (P7) developmental stage, insects were harvested and used in the study.

Collection of haemolymph and tissue protein samples

Larval haemolymph was collected by cutting prolegs and stored in cold test tubes. Then, haemolymph was centrifuged at 10,000 rpm for 10 min at 4℃ in order to remove hemocytes and cell debris, and the supernatant was stored at -70℃ until used. From the last instar larval stage to 7 days after pupal stage of *Papilio xuthus*, fat body, epidermis, cuticle and intestine was excised and washed with Ringer solution. Each same weight tissue sample was blended with homogenization buffer (5 mM tris, 38 mM glycine, pH 8.4). After centrifugation for 10 minutes at 15,000 x *g*, protein samples were collected and kept at -70℃.

Identification of transferrin

Native PAGE was conducted on 7.5% polyacrylamide gel as described by Davis [6]. After electrophoresis, Ferene S staining was performed as described by Chung [5] to identify the iron atoms contained in transferrin. The staining solution consisted of a mixture of 0.75 mM 3-(2-pyridyl)-5,6-bis(2-(5-furylsulfonic acid))-1,2,4-triazine, disodium salt (Ferene S) and 15 mM thioglycolic acid in 2% (v/v) acetic acid, and it was prepared just before use.

Purification of transferrin

Haemolymph (1.25 ml) was added in buffer A [0.1 M NaH_2PO_4, 0.15 M NaCl, 1 mM benzamidine, 0.5 mM PTC (phenylthiocarbamide), 5 mM EDTA, 5 mM sodium bisulfite, 1 mM TLCK (N-á-tosyl-L-lysinechloromethyl-ketone), pH 7.0] supplemented with 1.25 ml of 3.7 M KBr. Buffer A was added to give a final volume of 5 ml. The sample was centrifuged at 105,000 x *g* for 16 hr at 4℃ in Centrikon T-2190 ultracentrifuge. After centrifugation, the pellet was resuspended with buffer A and heated at 75℃ for 30 min. The supernatant was dialyzed against buffer B (25 mM sodium tetraborate, pH 9.0). Dialyzed sample were applied onto superose 6 HR 10/30 column using a FPLC at a flow rate of 0.15 ml/min, with 1 ml fraction. Then fractions containing transferrin were identified with Ferene S staining [5] after SDS-PAGE and Native PAGE. The molecular weight of transferrin was determined with 12% SDS-PAGE described by Laemmli [10].

Amino acids composition analysis of transferrin

Purified transferrin was hydrolyzed in vacuum with 6 N HCl for 24 hr. The hydrolyzed sample is applied to Biochrom 20 auto amino acid analyzer (Amersham Bioscience) for determining of amino acid composition.

Immunodiffusion and Rocket immunoelectrophoresis

To make Px (*Papilio xuthus*) transferrin antibody, the purified Px transferrin recovered from FPLC was emulsifieded with an equal volume of Freund's complete adjuvant (0.5 ml) and injected into a rabbit subcutaneously. Five injections were given for raising the polyclonal antibody against Px transferrin. One mililiter each sample were given two times at 2 days interval after the first injection, and the fourth injection was made 2 weeks later. To boost immunization, Freund's incomplete adjuvant (0.5 ml) was throughly mixed and injected 2 weeks after the fourth injection. One weeks after the final injection, antiserum against Px transferrin were prepared and used for immuno-diffusion and rocket immuno-electrophoresis assay described by Ouchterlony [15] and Axelsen and Svendsen [1] with minor modification, respectively. Two analytical methods are often used as quantitative tool for serum proteins which involves electrophoresis of antigen into a gel containing antibody. After the hardening of 1% agarose gel on glass plate, the antibody was spotted on the center well and extracted protein samples from tissues were spotted at circumference wells of center. The plate was incubated at 30℃ for 2 day, and soaked in 0.15 M NaCl solutions for one day. After washing the plate with tap water for 12 hr, the gel was stained with Coomassie brilliant blue R 250. For determining the changes of transferrin quantity during developmental

stage, extracted protein samples were loaded on the wells in agarose gel contained anti-transferrin antibody and electrophoresed at 200 V for 4 hr. The quantification of each peak was followed by the instruction of ImageQuant 5.2 from manufacturer.

Western blot

Electro transfer from non-denaturing and SDS gels was conducted at 90 V for 1 hr using semi-dry system (Bio-Rad). To identify the transferrin, anti Px transferrin and human anti transferrin (Cat. No. ab1223, Abcam, UK) were used as the first antibody and peroxidase conjugated anti-rabbit goat antibody (Bio-Rad) was used as the second antibody.

Results and Discussion

Characterization of transferrin during metamorphosis of *Papilio xuthus*

Papilio xuthus transferrin (Px transferrin) was purified and its activity was analyzed after obtaining haemolymph during metamorphosis with ferene S staining method and 7.5% native gel electrophoresis. In the haemolymph during the metamorphosis, the activity of the transferrin appears in two bands, designated Tf-1 and Tf-2. The activity of Tf-1 has only shown in last instar larval stage, but the activity of the Tf-2 shows in all developmental stages (Fig. 1). To purify the transferrin, the haemolymph were applied on superose-6 HR column with the FPLC system after fractionation using ultracentrifugation. The transferrin activity was detected in 13, 15, and 21 fractions of gel filtration chromatography (Fig. 2). Each freeze-dried fractions were applied into non-denaturing gel for checking transferrin activity and identifying of transferrin with Ferene S staining. Only no. 15 fraction was shown to have an activity of the transferrin with single band in non-denaturing gel (Fig. 3). Even though the fraction 21 showed iron-containing protein, ferritin, which is another form of ferrous

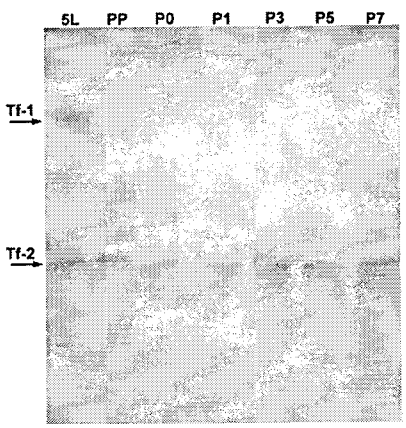

Fig. 1. Transferrin activity patterns of haemolymph from *Papilio xuthus* during metamorphosis. (5L: last instar larvae, PP: prepupae, P0: just after pupation, P1: 1 day after pupation, P3: 3 days after pupation, P5: 5 days after pupation, P7: 7 days after pupation). Non-denaturing PAGE gel was stained with ferene S to show activity of iron containing protein.

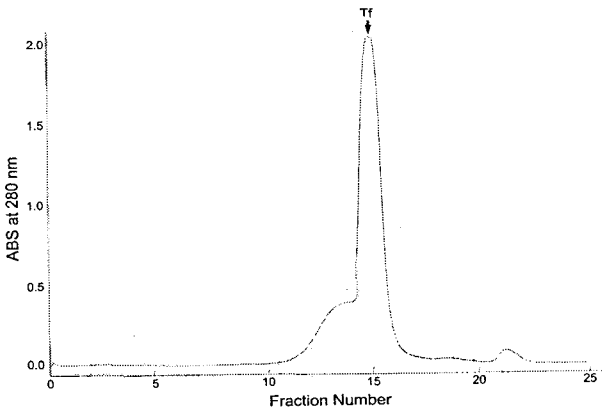

Fig. 2. Elution profile of transferrin from FPLC chromatography with Superose-6 HR 10/30 column after ultracentrifugation. The column was eluted with buffer B (25 mM sodium tetraborate, pH 9.0) at the flow rate of 0.15 ml/min. Fractions (1 ml each) were monitored at 280 nm (Tf indicates transferrin).

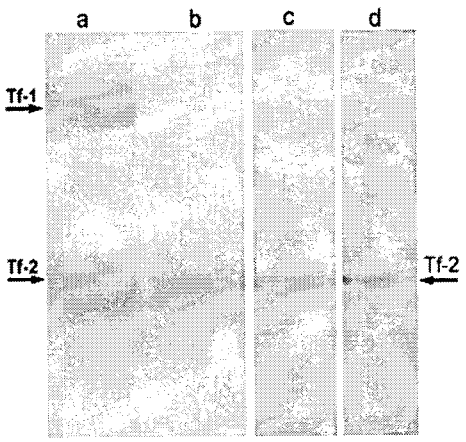

Fig. 3. Transferrin from the last instar larval haemolymph of *P. xuthus*. (a) total haemolymph and (b) eluted fraction Nr. 15. from superose-6 gel chromatography in non-denaturing gel stained with ferene S. (c) eluted fraction Nr. 15 in non-denaturing gel stained with Commassie brilliant blue. (d) Immunoblotted transferrin with antibody against transferrin which is prepared with eluted fraction no. 15.

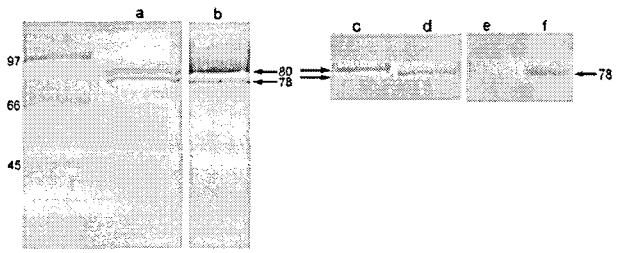

Fig. 4. Identification of transferrin from the last instar larval haemolymph of *P. xuthus*. (a) Two subunits were identified as 80 and 78 kDa in SDS-PAGE. (b) Western analysis of purified transferrin with raised antibody against transferrin. (c, d) Reapplied of excised two bands, 80 and 78 kDa subunit, from the gel into SDS-PAGE. The gel was stained Coomassie blue. (e, f) Immunoblot analysis of 80 (lane e) and 78 kDa(lane f) with specific antibody against with human transferrin. Only the 78 kDa subunit was shown the cross-reactivity with human transferrin-antibody.

protein and has a molecular weight of 45 kDa in many organisms including insects (data not shown). The Tf-2 was identified as transferrin which has a molecular weight of 78 and 80 kDa (Fig. 4). These data suggest that Px transferrin, which is consisted of two subunits. In order to identify the 'active' subunit, two bands were excised and eluted from the gel and reapplied to PAGE. Western analysis with antibody against human tansferrin shows that small subunit, 78kDa, might be have more homologous to human transferrin than large 80 kDa subunit (Fig. 4). Identity of large subunit would be analyzed in detail whether essential component to have transferrin activity or a kind of tightly binding protein to transferrin. The amino acids composition of Px transferrin-2 showed rich in aspartic acid (11.3%), valine (10.8%), leucine (10.6%) and glutamic acid (9.4%) (Table 1.). Significantly, Px transferrin-2 has more number of aspartic acid and glutamic acid than transferrins from the other insects.

Transferrin has a molecular mass of approximately 80 kDa in vertebrates [16]. In insects, cockroach transferrin [8] have a molecular mass of 78 kDa to similar vertebrates and *Manduca sexta* transferrin is 77 kDa [17]. In case of *Sarcophaga* molecular mass of transferrin is 65 kDa [9]. In this study we have purified the transferrin-2 from *P. xuthus* with molecular mass of 78 and 80 kDa subunit, which is similar to it in the other insects (Figs. 2, 3). Amino acid composition of *M. sexta* haemolymph transferrin was rich in Gly, Glu, Asp [7] and in this study, *P. xuthus* transferrin also showed abundance of Asp, Val, Leu, and Glu (Table

Table 1. Amino acid composition of transferrin of *P. xuthus* with that of other insects (Unit: %)

Amino acid	Citrus Swallowtail butterfly	Lubber grasshopper	Flesh fly	Yellow fever mosquito	Hawaiian Fruitfly	Fruitfly
	Papilio xuthus	*Romalea microptera*	*Sarcophaga peregrina*	*Aedes aegypti*	*Drosophila silvestris*	*Drosophila melanogaster*
Ala (A)	4.2	9.8	7.3	9.3	10.8	10.5
Arg (R)	4.0	4.8	4.1	5.8	6	6.4
Asn (N)	0.0	5.6	6	4.9	3.3	3.9
Asp (D)	11.3	6.8	5.9	7.4	6.4	6.2
Cys (C)	0.3	3.6	3.2	3.2	3.5	3.3
Gln (Q)	0.0	2.9	4	4.6	5	6.4
Glu (E)	9.4	4.8	8.1	5.8	7.5	7.2
Gly (G)	5.7	7.5	5.1	6.5	4.6	4.8
His (H)	5.7	2.6	2.1	1.3	2.7	2
Ile (I)	6.3	3.3	4.3	3.9	4.7	5
Leu (L)	10.6	10.3	8.4	8.4	9.9	9
Lys (K)	6.0	6.7	9.7	7.7	6.3	6.9
Met (M)	0.3	0.7	2.4	2.2	1.4	0.6
Phe (F)	5.1	3.1	3	4.1	2.8	3.1
Pro (P)	6.2	4.9	4.6	4.4	4.7	4.7
Ser (S)	3.9	6.6	5.2	4.9	5.7	5.5
Thr (T)	5.5	3.6	4.6	3.2	4.1	4.2
Trp (W)	0.0	1	0.5	0.5	0.8	0.5
Tyr (Y)	4.8	4.1	4.5	3.6	4.1	3.6
Val (V)	10.8	7.4	7	8.2	5.8	6.2
M.W (kDa)	78/80	80.5	71.1	70.6	71.5	71.8
# of AA	?	731	629	633	637	641

1). It could be speculated that increased number of acidic amino acids may make *P. xuthus* transferrin stable in urea and high temperature condition by increased number of ion-pair.

Tissue specific distribution of the transferrin.

With specific antibody against the purified transferrin, immuno-diffusion tests were performed to screen the transferrin in several tissues. A clear positive reaction showed when purified transferrin applied into the immuno-diffusion gel from the fat body, haemolymph and epidermis. Also slight positive reaction showed in intestine at the prepupal stage (Fig. 5). The amounts of transferrin in haemolymph during metamorphosis were compared quantitatively using rocket immuno-electrophoresis [1]. At the stage of 3 and 5 days after pupation, the single narrow peak showed at least 2 fold higher than that at the stage of 7 days after pupation (Fig. 6). In the case of fat body, the concentration of transferrin was decreased from prepupal to the 5 days after pupation and keep it in the stage of 7 days after pupation. The highest concentration of transferrin in the fat body showed in the prepupal stage during the developmental course (Fig. 7).

Comparing the haemolymph proteins, especially transferrin, from the developmental stages in housefly and tobacco hornworm, it shows high expression and the most broad distribution across the all organs at the last instar larval stage [4,7]. Here, we prepared specific antibody against transferrin and used immuno-diffusion and rocket

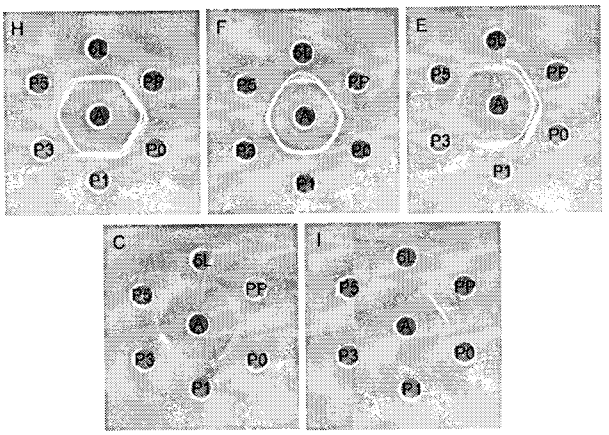

Fig. 5. Tissue specificity of transferrin during metamorphosis of *P. xuthus*. (A: Anti-transferrin, H: Haemolymph, F: Fat body, E: Epidermis, C: Cuticle, I: Intestine, 5L: last instar larval, PP: prepupal, P0: just after pupation, P1: 1 day after pupation, P3: 3 days after pupation, P5: 5 days after pupation)

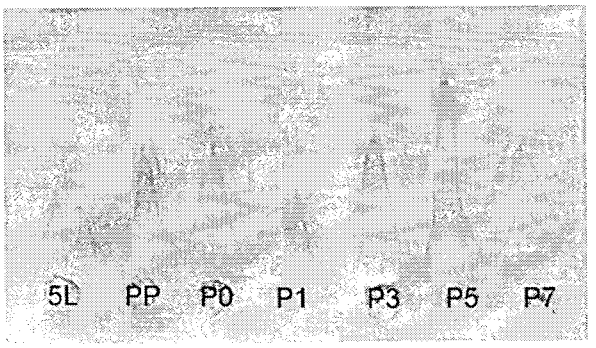

Fig. 6. Quantitative analysis of transferrin in the haemolymph during metamorphosis of *P. xuthus* with rocket immunoelectrophoresis. (5L: last instar larvae, PP: prepupae, P0: just after pupation, P1: 1 day after pupation, P3: 3 days after pupation, P5: 5 days after pupation, P7: 7 days after pupation)

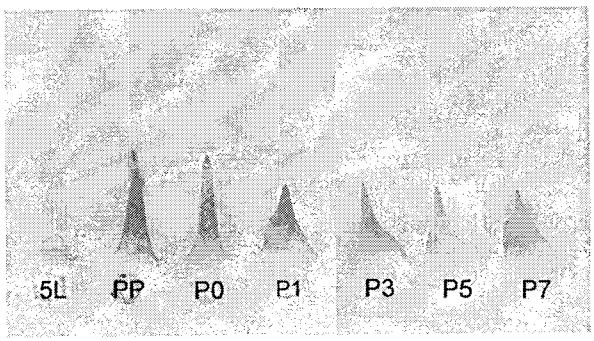

Fig. 7. Quantitative analysis of transferrin in the fat body during metamorphosis of *P. xuthus* with rocket immunoelectrophoresis. (5L: last instar larvae, PP: prepupae, P0: just after pupation, P1: 1 day after pupation, P3: 3 days after pupation, P5: 5 days after pupation, P7: 7 days after pupation).

immuno-electrophresis analysis to compare the distribution and quantity of transferrin. In citrus swallowtail butterfly, *P. xuthus*, the amount of transferrin was significantly reduced from the stage of 7 days after pupation. Transferrin is highly expressed from the last instar larval to 5 days after pupation stage without any detectable changes. Interestingly, in the fat body, small amount of transferrin and highest amount of transferrin were detected at the last instar larval and prepupal stage, respectively. After the stage just after pupation, the amount of transferrin was reduced with significant ratio until the stage of 3 days after pupation (Figs. 6,7). These results suggest that the changes of transferrin concentration may not affect the development of insects.

In the insects, transferrin is the form of storage and secretion for iron. It helps to stabilize and maintain the ho-

meostasis of the living organism. Transferrin is absorbed, transported and secreted from the intestine and mainly stored in the haemolymph. It is also stored in the fat body and acts as a storage tube for iron. Here, the distribution of the transferrin across the tissues is assayed with specific antibody using immuno-diffiusion analysis. Transferrin is localized in the haemolymph, fat body, epidermis at the all stage during development whereas it is undetectable in cuticle at any stages. In intestine, it is found only in prepupal stage during the development (Fig. 5). Quantitative analysis shows that the amount of transferrin is increased in haemolymph from the 5 days after pupation even though in fat body from prepupa is decreased. In order to define the quantitative changes and activities of transferrin, the metabolism of iron after uptake in insects should be analyzed with intake of other heavy metals.

Acknowlegment

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초록 : 호랑나비 유충 혈림프 Transferrin의 특성과 분포

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Transferrin은 저장된 철분자를 운반하고 살아있는 유기체에서 철의 항상성을 유지한다. 호랑나비의 종령 유충 혈림프내 철 운반 단백질인 transferrin을 KBr 밀도구배 초원심분리와 Superose 6 HR을 이용한 fast protein liquid chromatography법으로 분리 정제하였으며, transferrin의 조직에 따른 분포는 면역화학적 방법에 따라 확인하였다. 정제된 transferrin의 아미노산 조성은 아스파르트산(Asp), 발린(Val), 루이신(Leu), 글루타민(Glu)이 많이 존재하였으며, subunit의 분자량은 78, 80 kDa으로 확인되었다. Immuno-diffusion을 통해 각 조직에서 분포를 확인한 결과 혈림프와 지방체에서 transferrin이 전시기에 걸쳐 뚜렷한 동질성을 나타냈다. 또한 rocket immuno-electrophresis법에 의해 transferrin의 양적 분포를 살펴보면 혈림프에서 용화 3일과 용화 5일에 증가하였으며, 지방체에서는 전용기와 용화직후에 양적인 증가를 나타냈다.