

# Separation and Purification of Ovotransferrins from the Six Poultry Species

## 6種家禽オボトランスフェリンの分離と精製

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### 要 旨

The methods for the preparation of gram-scale ovotransferrins of six poultry species, chicken, duck, guinea fowl, Japanese quail, ostrich and turkey were studied for the comparative research on their functional properties. The combination of two-step ethanol fractionation applied for chicken ovotransferrin and ion-exchange chromatography were employed for the purification. The optimal ethanol concentration for the first step of the fractionation to remove ovalbumin was 43% for the poultry species other than duck which required a lower ethanol concentration. For the second step to precipitate ovotransferrins, 60% ethanol was optimal for the five poultry species other than duck. The optimal ethanol concentration for duck was 58%. The suitable condition for the elution of purified ovotransferrin through DEAE-column was also determined for each poultry species. The purity of the poultry ovotransferrins obtained was more than 99.6% estimated by densitometry on SDS-PAGE gel. The yields were around 1 g to 7 g/L of egg whites depending on the poultry species. Neutral sugar contents of purified ovotransferrins were from 1% to 2.8% for the six poultry species and the highest for ostrich.

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## Introduction

Chicken (Chi) ovotransferrin (OTrf) is one of the major components comprising about 12% of egg white proteins and contributes to the useful functional properties in food, such as solubility, gelling properties and foaming ability of egg white proteins. OTrfs are also found in poultry as well as Chi such as duck (Duk), guinea fowl (Gui), Japanese quail (Jpq), ostrich (Ost) and turkey (Tky) in varying contents from 2% to 15% of egg white proteins (Feeney *et al.*, 1960; Burley and Vandehra, 1989). However, detailed information about properties of such poultry OTrfs is not yet fully available, and we have started comparative study on the thermal stability of poultry OTrfs (Suzuki *et al.*, 2022). For such study, therefore, efficient separation and purification of the six poultry OTrfs in gram-scale are essential for further research on the comparative studies to exploit the potentials of OTfs and poultry egg whites.

A variety of separation methods have been reported for Chi OTrf; salting out with ammonium sulphate, ethanol fractionation, ion-exchange chromatography, the combination of the methods above (Azari and Baugh, 1967; Awadé *et al.*, 1994; Vachier *et al.*, 1995; Abeyrathne *et al.*, 2013) or metal-affinity chromatography taking advantage of metal-binding by Chi OTrf (Al-Mashikhi and Nakai, 1987; Wu and Acero-Lopez, 2012). However, only limited information is available on the preparation of the other poultry OTrfs; the combination of cation- and anion-exchange methods for Jpq OTrf (Clark *et al.*, 1963), salting out followed by gel-filtration and anion-exchange chromatography for Ost OTrf (Ciuraszkiewicz *et al.*, 2006) and ion-exchange chromatography with

or without salting out, or the combination of gel filtration and ion-exchange chromatography for Tky OTrf (Clark *et al.*, 1963; Ciuraszkiewicz *et al.*, 2006). Some of these methods suffer some problems related to the yield and purity of OTrf, as well as scale-up issues. An appropriate combination of the methods described above, therefore, should lead to the promising procedures commonly applicable to the six poultry species to obtain gram-scale purified poultry OTrfs.

In the present paper, the applicability of the industrial separation method by ethanol fractionation reported for Chi OTrf (Ko and Ahn, 2008) was tested for the other poultry OTrfs and the optimal conditions and the procedures to separate crude OTrfs were determined. The purification of the poultry OTrfs from the crude fraction by ion-exchange chromatography was also conducted. The poultry OTrfs as glycoproteins were partly characterized by their neutral sugar contents.

## Materials and Methods

### *Separation of crude OTrfs by ethanol fractionation in poultry*

Chi, Duk, Gui, Jpq, Ost and Tky eggs were obtained from the suppliers as described previously (Suzuki *et al.*, 2022). The poultry OTrfs were separated from egg whites essentially by the method for Chi OTrf according to Ko and Ahn (2008). In principle, the method is consisted of 2-step cold ethanol fractionation: at the first step, the precipitation (Ppt) and removal of ovalbumin (OVA) from egg white proteins by ethanol after the formation of OTrf-Fe(III) complex and at the second step, the precipitation of OTrf-Fe(III) by the increase of

ethanol concentration in the supernatant (Sup) obtained by the first step. The scheme of the separation of crude OTrfs in poultry is shown in Fig. 1. As for Chi OTrf, the concentrations of ethanol were 43% and 59% at the first and second steps, respectively (Ko and Ahn, 2008). Incidentally, the formation of OTrf-Fe(III) complex was carried out to prevent the denaturation of OTrf during ethanol treatment according to Ko and Ahn (2008). Then, applicability of the method for Chi OTrf to the other poultry OTrfs was examined by altering the ethanol concentrations for the first and the second steps of the fractionation.

At the first step, cold ethanol was added to the diluted egg white solutions to give 37%, 40%, 43%, 47% or 50% in the mixture. The supernatant and precipitate obtained after centrifugation were subjected to SDS-PAGE analyses on the efficiency of ovalbumin removal from the supernatant and the yield of poultry OTrfs. The optimal ethanol concentration at the first step was determined for each poultry based on the analyses. To the supernatant on each poultry obtained with the optimal ethanol concentration at the first step, cold ethanol was gradually increased to give 56%, 58%, 60%, 62% or 64% in the mixture at the second step. The precipitate and supernatant after centrifugation for each poultry were subjected to SDS-PAGE analysis to determine the optimal ethanol concentration that would give the maximum yield of OTrf while reducing contaminants in the precipitate. The precipitate obtained by the second step of the ethanol fractionation under the optimal condition was dissolved in water for each poultry and the solution was subjected to the procedures for the removal of Fe(III) ions from poultry OTrfs and the solution according

to Ko and Ahn (2008) as shown in Fig. 1. The crude OTrfs in poultry were recovered from the solution by lyophilization after dialysis against water.

#### ***Purification of poultry OTrfs by anion-exchange chromatography***

Approximately 2 mL of 20 mg/mL solution of crude OTrfs in poultry was applied at a time to a column (HiTrap DEAE FF, 5 mL, GE Healthcare, Tokyo) and eluted by a linear gradient of NaCl concentration in 0.2 mol/L Bis-Tris HCl buffer (pH 6.4) as described by Suzuki *et al.* (2022). The ranges of NaCl concentration in the gradient were from zero to 0.3 mol/L depending on the poultry species. Protein and NaCl concentration in the eluate were monitored by a UV detector (UV-900, GE-Healthcare) at 280 nm and an ion monitor (C-900, GE-Healthcare), respectively. The fractions containing OTrf determined by SDS-PAGE analysis were pooled, dialyzed against water and lyophilized.

#### ***SDS-PAGE analyses***

SDS-PAGE was performed using 12.5% polyacrylamide gel (c-PAGEL CHR125L, ATTO, Tokyo) according to Laemmli (1970). The marker (EzStandard, ATTO) was used for the estimation of molecular weights of proteins. Protein was stained by Coomassie Brilliant Blue after electrophoresis using a staining solution (EzStain Aqua, ATTO). The dried gel was scanned for densitometry using the Gel tool of ImageJ (NIH, Maryland, U.S.A.) to estimate the purity of the poultry OTrfs obtained after the chromatographic separation. PAS staining was performed using a kit (Glycoprotein Western Detection Kit, Takara Bio, Tokyo) on the PVDF membrane after transfer of proteins from SDS-PAGE gel by an apparatus (eBlot, GenScript, New Jersey, U.S.A.).

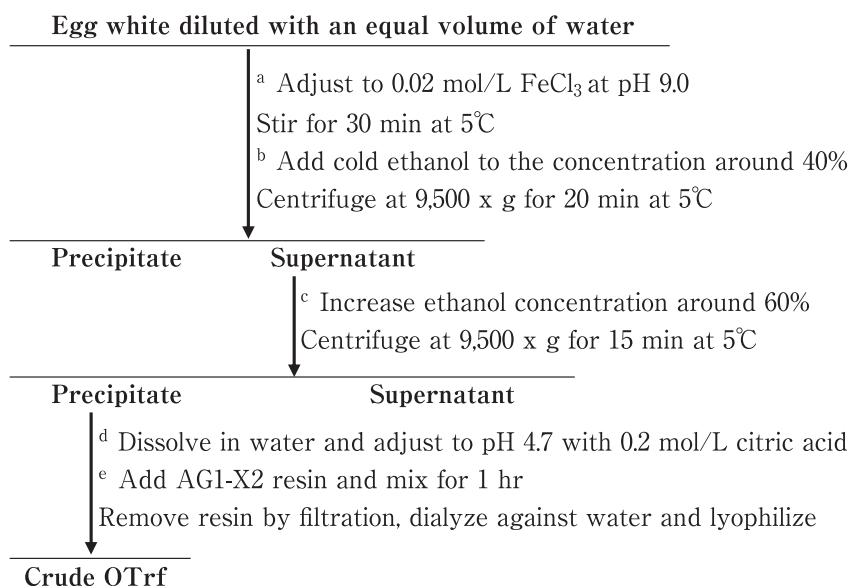


Fig. 1. Scheme for the separation of crude poultry OTrfs by ethanol fractionation.

a: Formation of OTrf-Fe(III) complex; b: The first step of ethanol fractionation; c: The second step of ethanol fractionation; d: Dissociation of Fe (III) ions from OTrf; e: Removal of Fe (III) ions by ion-exchange resin. The optimal ethanol concentrations at the first<sup>b</sup> and the second<sup>c</sup> steps depend on the poultry species as described in the text.

### Other methods

The neutral sugar conjugated with poultry OTrfs was determined by the phenol-sulfuric acid method according to Dubois *et al.* (1956) using mannose as a standard. Protein was determined by bicinchoninic acid assay (#23225, Thermo Fisher Scientific, Massachusetts, U.S.A.) using bovine serum albumin as a standard.

The statistical analysis was performed using multiple comparisons in SPSS software, and the significance level was set at 1%. Values are expressed as mean  $\pm$  SD (n=3).

## Results and Discussion

### Separation of crude OTrfs by ethanol fractionation in poultry

The molecular weights of the major egg white proteins, ovalbumin (OVA), OTrf and ovomucoid (OVM) of the six poultry species including Chi can be estimated based on their sequences (NCBI database). The NCBI GenBank accession numbers of the protein sequences are AAB59956 (OVA), NM\_205304 (OTrf) and NP\_001295423 (OVM). The contents of the six poultry OTrfs in the egg white proteins have been reported (Feeney *et al.*, 1960; Clark *et al.*, 1963) and the compositions of the other major proteins for several poultry species are largely known

(Burley and Vadehra, 1989; Quan and Benjakul, 2019; Huang and Lin, 2011). Moreover, the SDS-PAGE patterns of egg white proteins have been reported on the six poultry species (Miguel *et al.*, 2005; Utratuna *et al.*, 2017). Based on such information, stained bands for OVA, OTrf and OVM of the six poultry species on SDS-PAGE gels were labelled as shown in Figs. 2 and 3. At the first step of ethanol fractionation up to 43% ethanol, most Chi OTrf was obtained in the supernatant with the contaminants, mainly OVM (lanes 1-3 in Fig. 2: Chi) and the amount of Chi OTrf significantly decreased by the increase of ethanol (lanes 4-5) to 47% or higher. Chi OTrf was slightly lost in the precipitate and its amount clearly increased at 47% or higher ethanol concentration. Chi OVA, most abundant egg white protein, did not contaminate in the supernatant but appeared in the precipitate (lanes 6-10) at any ethanol concentration tested. These findings clearly support that 43% ethanol in the first step was appropriate to efficiently remove most OVA from Chi OTrf fraction as pointed out by Ko and Ahn (2008). Then, the applicability of the method for Chi to the other poultry species was examined. Duk OTrf, a minor protein comprising only 2% of egg white proteins (lane 11 in Fig. 2: Duk; Feeney, *et al.*, 1960), mainly obtained in the supernatant (lanes 1-5) although the amounts decreased at 43% or more ethanol in the mixture (lanes 3-5). Duk OVA was almost completely removed from the supernatant and fractionated in the precipitate, although the supernatant was intensely contaminated with OVM at any ethanol concentration tested. From these results, 40% ethanol was supposed to be optimal for Duk at the first step (lane 3). As for Gui and Jpq, OTrfs were fractionated in the supernatant (lanes 1- 5

in Fig. 2: Gui and Jpq), although their yields decreased at 47% or more ethanol in the mixture (lanes 4-5). OVAs were efficiently removed from the supernatants into the precipitates at any ethanol concentration tested. Then, 43% ethanol was supposed to be optimal for Gui and Jpq considering the noticeable amounts of recovered OTrfs and less contaminated proteins such as OVMs and 50 kD components (lane 3). The latter 50 kD protein for Jpq is assumed to be ovoinhibitor as reported by Takahashi *et al.* (1992). Ost and Tky OTrfs were mostly recovered in the supernatant up to 43% (lanes 1-3 in Fig. 2: Ost and Tky), though they were considerably lost at 47% and 50% ethanol concentration (lanes 4-5). However, the contamination of OVA was observed at 37% and 40% ethanol concentration for both Ost and Tky (lanes 1-2). OVM inevitably appeared in the supernatant at any ethanol concentration tested. Then, 43% ethanol in the mixture for the first step (lane 3) was supposed to be optimal for Ost and Tky. From these results, 40% for Duk and 43% ethanol for Gui, Jpq, Ost and Tky as well as for Chi were supposed to be optimal for the first step of ethanol fractionation. However, the yield of OTrfs for Gui, Jpq and Tky should be improved by the finer adjustment of ethanol concentration since OTrfs partly lost in the precipitate at 43% ethanol (lane 8, Fig. 2: Gui, Jpq and Tky).

In the second step, the supernatants obtained by the optimal ethanol concentration at the first step were used. Chi OTrf was mostly fractionated into the precipitate by more than 56% ethanol in the mixture (lanes 7-11 in Fig. 3: Chi). Some OTrf was lost in the supernatant at 56% and 58% ethanol in the mixture (lanes 1-2) and appreciable amount of OVM was detected

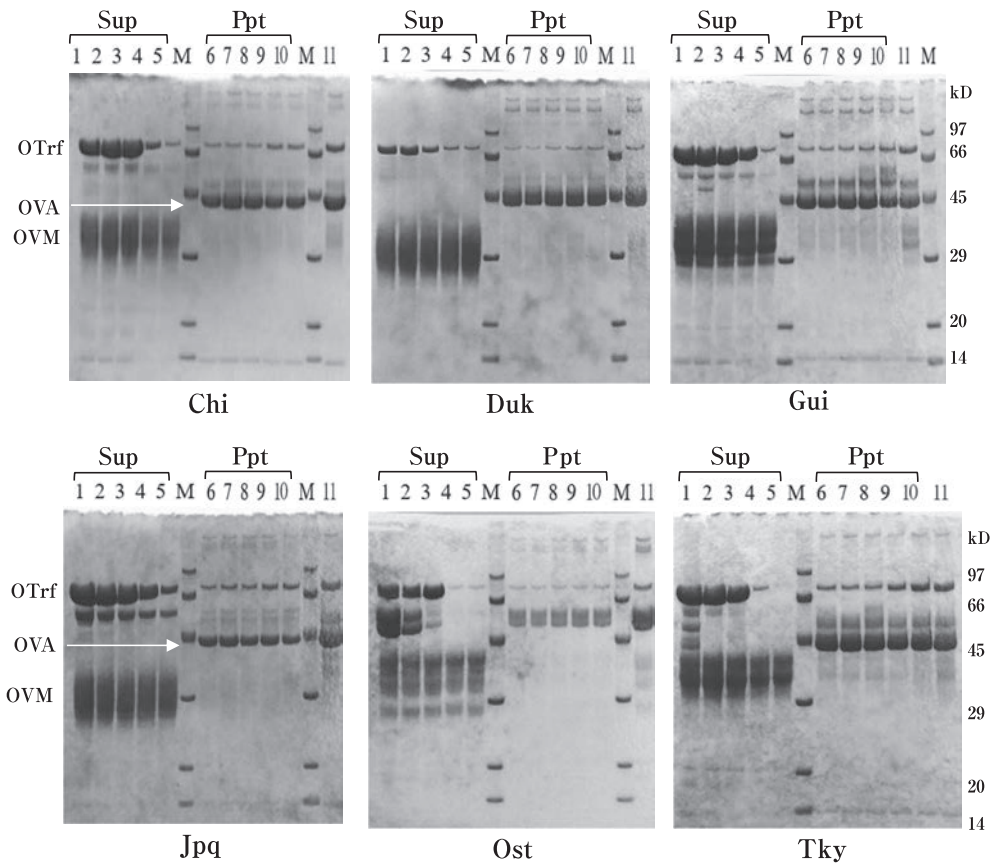


Fig. 2. SDS-PAGE patterns of the supernatant and the precipitate obtained by the first step of ethanol fractionation.

Poultry species are shown above the electrophoregrams.

Sup: supernatant; Ppt: precipitate.

Lanes 1 and 6: 37% ethanol; lanes 2 and 7: 40% ethanol; lanes 3 and 8: 43% ethanol; lanes 4 and 9: 47% ethanol; lanes 5 and 10: 50% ethanol; lane 11: egg white; M: molecular weight marker.

Protein bands for major egg white proteins, OTrf, OVA and OVM were labelled based on the information in the literature about their molecular weights and the composition of egg white proteins of poultry species.

in the precipitate at 62% and 64% ethanol (lanes 10-11). Thus, 60% ethanol was appropriate to recover OTrf in the precipitate (lane 9), although the fractions still contaminated with some OVM. Duk OTrf was well recovered in the precipitate by 58% or more ethanol in the mixture (lanes 8-11 in Fig. 3: Duk). Some of Duk OTrf was lost

in the supernatant by 56 and 58% ethanol in the mixture (lanes 1 and 2). So, 60% or more ethanol in the mixture supposed to preferable for the recovery of Duk OTrfs and for the removal of Duk OVM. However, the precipitate obtained by 60% or more ethanol in the mixture was not easily solubilized probably due to the



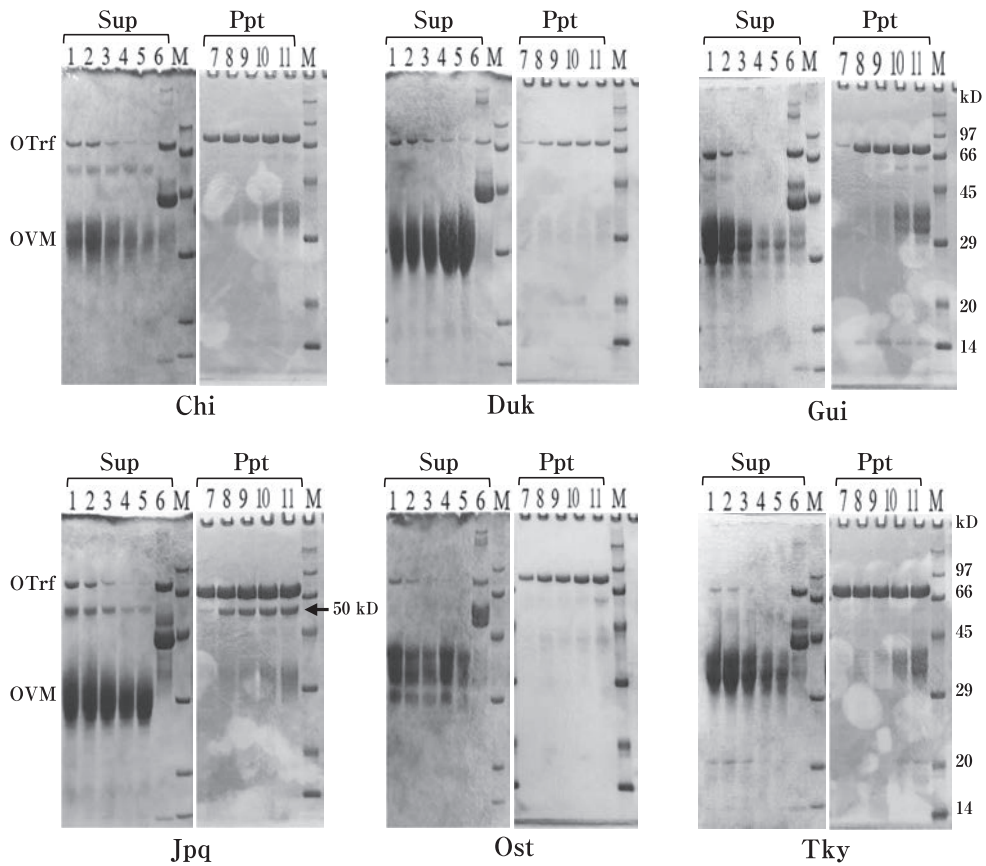


Fig. 3. SDS-PAGE patterns of the supernatant and the precipitate obtained by the second step of ethanol fractionation.

Poultry species are shown above the electrophoregrams. Sup: supernatant; Ppt: precipitate. Lanes 1 and 7: 56% ethanol; lanes 2 and 8: 58% ethanol; lanes 3 and 9: 60% ethanol; lanes 4 and 10: 62% ethanol; lanes 5 and 11: 64% ethanol; lane 6: egg white; M: molecular weight marker.

Protein bands for major egg white proteins, OTrf, OVA and OVM were labelled based on the information in the literature about their molecular weights and the composition of egg white proteins of poultry species.

denaturation. Then, 58% ethanol was adopted for Duk in the second step of the fractionation (lane 8). Gui, Jpq, Ost and Tky OTrfs were obtained mostly in the precipitate (lanes 7-11 in Fig. 3: Gui, Jpq, Ost and Tky) but slightly lost in the supernatant at 56% and 58% ethanol (lanes 1-2). The contamination with OVMs were apparent at 62% and 64% ethanol (lanes 10-11). Then, the

optimal ethanol concentration for Gui, Jpq Ost and Tky should be 60% (lane 9) to reduce the loss of OTrfs and the least contamination with OVMs. The contamination of 50 kD component (lane 9 in Fig. 3: Jpq), probably ovoinhibitor, in the precipitate for Jpq even at 60% ethanol should be taken into consideration in the process of purification. The efficient removal of OVM

from Ost OTrf fraction was also noted. In summary on the second step, 58% ethanol for Duk and 60% ethanol were supposed to be optimal for the other four poultry species as well as for Chi.

In conclusion, the optimal ethanol concentration at the first step was 40% for Duk and 43% for Gui, Jpq, Ost and Tky as well as for Chi. At the second step, 58% ethanol for Duk and 60% ethanol were optimal for the four species as well as for Chi to recover most poultry OTrfs in the present study, although 59% ethanol was used for Chi by Ko and Ahn (2008).

#### *Purification of poultry OTrfs by anion-exchange chromatography*

The poultry OTrf fractions obtained by the ethanol fractionation were crude and still contained small amounts of contaminants, mainly OVM and other proteins (lane 9 in Fig. 3: all poultry species). The chromatography was performed to isolate OTrfs and remove such contaminants. Representative chromatogram of crude Jpq OTrf was shown in Fig. 4.

Jpq OTrf was eluted in the range of 0.01 and 0.04 mol/L NaCl whereas the major contaminant, ovoinhibitor passed through the column. Chi, Duk, Gui and Tky OTrfs were eluted in the range of 0.01 to 0.11 mol/L NaCl, 0.01 to 0.06 mol/L NaCl, 0.01 to 0.07 mol/L NaCl and 0.01 to 0.06 mol/L NaCl, respectively (Fig. not shown). Ost OTrf did not bound, but contaminated proteins adsorbed to the column under the present pH condition. SDS-PAGE patterns of the six poultry OTrfs obtained after the chromatography are shown in Fig. 5. The densitometry of the gel revealed that the purity of the six poultry OTrfs was more than 99.6%. The yields of the poultry OTrfs were 7.4 g/L, 0.7

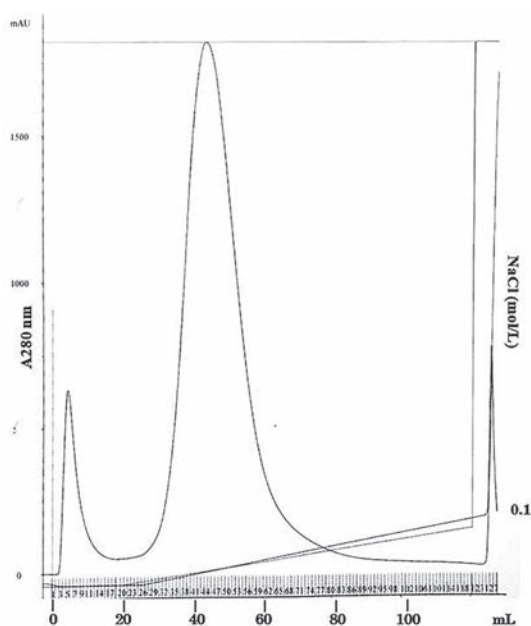


Fig. 4. DEAE ion exchange chromatogram of crude Jpq OTrf.

A DEAE Sepharose column (5 mL) was equilibrated with 0.2 mol/L Bis-Tris HCl buffer (pH 6.4). The sample solution (2 mL at a time, approximately 20 mg/mL) was applied and bound proteins were eluted by a linear gradient of NaCl concentration in the same buffer as described by Suzuki *et al.* (2022). The final NaCl concentration for elution was optimized depending on the poultry species. The fractions within the vertical lines were pooled as purified OTrf fractions.

g/L, 2.0 g/L, 2.7 g/L, 0.6 g/L and 1.9 g/L of egg white for Chi, Duk, Gui, Jpq, Ost and Tky, respectively throughout the separation by ethanol fractionation and chromatography. Therefore, the combination of ethanol fractionation and ion-exchange chromatography described here enables to prepare gram-scale purified poultry OTrfs enough for the comparative research in their functional properties. Relatively low yields shown for Gui,



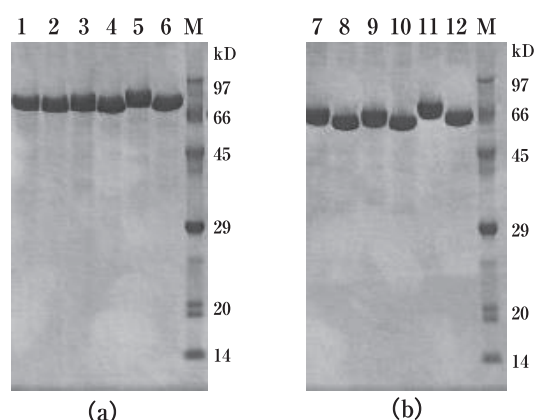


Fig. 5. SDS-PAGE patterns of purified poultry OTrfs.

3.2  $\mu$ g of purified protein was applied to each lane.

(a) Reduced samples; (b) Non-reduced samples.

Lanes 1 and 7: Chi; lanes 2 and 8: Duk; lanes 3 and 9: Gui; lanes 4 and 10: Jpq; lanes 5 and 11: Ost; lanes 6 and 12: Tky; M: molecular weight marker.

Jpq and Tky, whose contents of OTrfs were comparable to that of Chi (Feeney *et al.*, 1960; Clark *et al.*, 1963) should be mainly due to the loss in the precipitates at the first step. The molecular weights of purified OTrfs estimated based on the mobility of standard proteins and the samples were 73.9 kD, 71.9 kD, 72.5 kD, 72.5 kD, 79.7 kD and 73.9 kD for Chi, Duk, Gui, Jpq, Ost and Tky, respectively. The values for poultry OTrfs except for Ost OTrf were lower than those around 75.5 kD calculated based on their amino acid compositions in the database (NCBI database), whereas the molecular weight of Ost OTrf estimated by SDS-PAGE was slightly higher than 75.7 kD probably due to the conjugated carbohydrates described below.

#### *Carbohydrates conjugated with poultry OTrfs*

Chi OTrf and Duk OTrf are glycoproteins (Williams, 1968; Graham and Williams, 1975). For

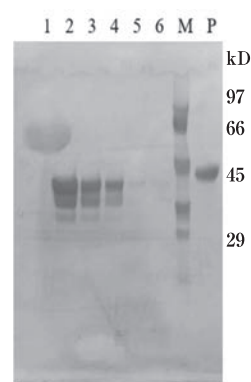


Fig. 6. PAS staining of Ost OTrf and OVM transferred to PVDF membrane.

Lane 1: 160  $\mu$ g of OTrf; lane 2: 6.4  $\mu$ g of OVM; lane 3: 3.2  $\mu$ g of OVM; lane 4: 1.6  $\mu$ g of OVM; lane 5: 0.6  $\mu$ g of OVM; lane 6: 0.3  $\mu$ g of OVM; M: molecular weight marker; P: horseradish peroxidase as positive control.

the confirmation, PAS staining was performed for the purified OTrfs on the PVDF membrane. Chi, Duk, Gui, Jpq and Tky OTrfs did not show detectable bands even at 160  $\mu$ g (Fig. not shown), but Ost OTrf exhibited distinct band at the same amount (lane 1, Fig. 6) although Ost OVM gave clear bands even at 1.6  $\mu$ g (lane 4).

These results suggest that the carbohydrate content of Ost OTrf is much higher than those of the other five poultry OTrfs but significantly lower than that of Ost OVM. Then, the neutral sugar contents of the purified poultry OTrfs were compared (Table 1). Chi, Duk and Tky OTrfs contained about 1% neutral sugar. The contents in Gui and Jpq OTrfs were slightly higher but below 2%, and Ost OTrf exhibit the highest content around 2.8%, supporting positive staining by PAS described above. Neutral sugar contents of Chi and Duk OTrfs reported are 0.8% and 1.9%, respectively (Williams, 1968;

Table 1. Neutral sugar conjugated with the purified poultry OTrfs.

OTrf	Neutral sugar (%)
Chi	1.05 ± 0.01
Duk	1.00 ± 0.06 n.s.
Gui	1.51 ± 0.03 **
Jpq	1.84 ± 0.03 **
Ost	2.79 ± 0.09 **
Tky	1.18 ± 0.01 n.s.

The contents are expressed as percentages of the protein amounts.

Statistically significant differences from chicken are shown by superscript. Values are expressed as mean ± SD (n=3).

n.s.: not significant; \*\*: p<0.01.

Graham and Williams, 1975), although no information is available so far about those of the other poultry OTrfs. It has been reported that the expression of Chi Trf gene in the oviduct leads to the synthesis of OTrf differing from serum Trf only in the conjugated carbohydrates. These facts suggest that the poultry OTrfs are supposed to be glycoproteins as well as Chi and Duk OTrfs. Moreover, their amino acid sequences are identical around the carbohydrate conjugation site (residue 473) of Chi or Duk OTrf (NCBI database; Williams *et al.*, 1982; Raws *et al.*, 1989). Our findings confirm that Gui, Jpq, Ost and Tky OTrfs are also glycoproteins with varying neutral sugar contents below 3%.

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