

## Comparison of the Amino Acid Sequences of Hen Plasma-, Yolk-, and White-Riboflavin Binding Proteins

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The amino acid sequence of hen egg yolk-riboflavin binding protein (yolk-RBP) was determined by conventional methods. The sequence was identical with that of hen egg white-riboflavin binding protein except that their carboxyltermini were different, that of yolk-RBP lacked 11 or 13 amino acid residues, while hen plasma-RBP had the same C-terminal sequence as white-RBP. This indicated that the C-terminal 11 or 13 amino acid residues in plasma-RBP might be cleaved off during the incorporation from the blood into the oocyte or in the yolk fluid.

Yolk-RBP had the same characteristics as white-RBP, such as N-terminal pyroglutamic acid, polymorphism in the amino acid sequence (Lys/Asn) at the fourteenth residue from the N-terminal end, carbohydrate chains attached to both Asn(36) and Asn(147) residues, and phosphate groups bound to some serine residues in the sequence of Ser(185) to Ser(197) as a cluster.

These results led us to the conclusion that yolk- and white-RBPs are bio-synthesized from the same gene in the different organs (liver and oviduct).

The carbohydrate composition of yolk-RBP was identical to that of plasma-RBP but different from that of white-RBP showing that the processing of the carbohydrate chains in the liver was different from that in the oviduct.

Egg yolk-riboflavin binding protein (yolk-RBP) is synthesized in the liver of the laying hen as are other yolk proteins. This protein as well as other yolk proteins are transported *via* the blood to the ovary, and deposited in the oocyte (1–3). Egg white-riboflavin binding protein (white-RBP) is synthesized in the oviduct (4). White-RBP is comprised of 219 amino acid residues and its sequence

has been determined (5). Egg yolk-, plasma-, and white-RBPs are thought to be products from the same gene (3, 6). However, we found slight differences in the amino acid compositions, especially in the content of glutamic acid between yolk- and white-RBPs (5). To clarify the difference in their amino acid compositions and to confirm whether both proteins are the products of the same gene, we examined the amino acid sequence of yolk-RBP. In the course of the study, we found that the carboxyl-terminal amino acid sequence of yolk-RBP was 11 or 13 residues shorter than that

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Abbreviations: RBP, riboflavin binding protein; RCM, reduced and carboxymethylated; PMSF, phenylmethane sulfonyl fluoride.

of white-RBP. Therefore, we also examined the C-terminal sequence of plasma-RBP.

## MATERIALS AND METHODS

**Materials**—Egg yolk-RBP and white-RBP were purified from fresh White Leghorn eggs as reported previously (5). Proteolytic enzymes used in this study for the determination of amino acid sequences were the same as those reported before (5). All solvents used were purified by distillation. Reagents were of analytical grade.

**Purification of Plasma-RBP**—Isolation of plasma-RBP from blood was performed according to the method of Miller *et al.* (7) as follows. Citrate buffer (0.5 M, pH 5.5) was immediately mixed with fresh blood in a ratio of 3 to 5 to avoid coagulation. The serine protease inhibitor, PMSF (100 mg in 10 ml acetonitrile per 1 L blood), was also added to the blood. After the removal of particulate materials by centrifugation for 10 min at  $5,000 \times g$ , the plasma fraction was subjected to ammonium sulfate fractionation. The precipitate which appeared at 45%  $(\text{NH}_4)_2\text{SO}_4$  saturation was removed by centrifugation for 10 min at  $5,000 \times g$ . The precipitate at 45–85%  $(\text{NH}_4)_2\text{SO}_4$  saturation was collected as a floating solid by centrifugation for 0.5 h at  $16,000 \times g$ . The precipitate was dissolved in water and dialyzed against distilled water. The pH of the dialyzed solution was adjusted to 5.5 with 1 M acetic acid and insoluble material was removed by centrifugation. To the supernatant, 20 g of buffered DEAE-cellulose (0.1 M sodium acetate, pH 5.5) was added. After washing the cellulose with the buffer, RBP was eluted with 1 M sodium chloride in the same buffer. This eluate was dialyzed against 0.1 M sodium acetate buffer, pH 5.5, and applied to a DEAE-cellulose (DE-52) column ( $2 \times 21$  cm) and eluted with a linear NaCl concentration gradient of from 0 M to 1 M in the buffer. The RBP fraction was purified by gel filtration on a Sephadex G-100 column ( $2.5 \times 133$  cm) with 0.02 M ammonium acetate. The yellow fraction was collected and lyophilized. The yield of RBP was 53 mg from 5 L blood and its purity was confirmed by polyacrylamide gel electrophoresis (Fig. 1S).

The following methods used in this study were described in our previous paper (5): Polyacrylamide gel electrophoresis, amino acid analysis,

sugar analysis, reduction, and S-carboxymethylation, separation of peptides, analysis of phosphoserine, enzymatic digestions, cleavage with cyanogen bromide and sequence analysis.

## RESULTS

**Compositions of Plasma-, Yolk-, and White-RBPs**—The amino acid and carbohydrate compositions of plasma-, yolk-, and white-RBPs are summarized in Table I.

These RBPs have similar amino acid compositions, but that of plasma-RBP was closer to that of white-RBP than that of yolk-RBP, especially with regard to glutamic acid and leucine contents. The carbohydrate compositions of both plasma-RBP and yolk-RBP were in accord with those of a complex type sugar chain and different from that of white-RBP.

**Amino Acid Sequence of Yolk-RBP**—The amino acid sequence of yolk-RBP was determined by the same method as used for sequence analysis of white-RBP (5). After reductive carboxymethylation and CNBr oxidation of yolk-RBP, the CNBr-peptides were separated into four fractions (I to IV) on Sephadex S-200 (Fig. 2S). The fractions were purified by HPLC (5). As shown in Fig. 3S, fraction I gave CNBr-peptide A, fraction III gave eight CNBr-peptides (B, B', C-1, C-2, D-1, D-2, E, and F), and fraction IV gave CNBr-peptides G and H, which were separated by paper electrophoresis. Fraction II was a mixture of incompletely cleaved CNBr-peptides. The amino acid compositions of the 11 CNBr-peptides thus purified are summarized in Table IIS. Peptides A, B, and B' contained carbohydrates and the amino acid composition of peptide B' coincided with the sum of those of peptides B and G. The absence of homoserine in peptides C-1 and C-2 showed that both peptides were derived from the C-terminal of the protein and their amino acid compositions were 13 or 11 residues shorter than the C-terminal CNBr-peptide of white-RBP, respectively.

The difference in the compositions of peptides D-1 and D-2 was by only one residue of lysine and aspartic acid as found in white-RBP. Peptide E was obtained as a mixture of several peptides having the same amino acid composition but different phosphate contents (Fig. 3S). Peptides C-1

TABLE I. Amino acid and sugar compositions of plasma-, yolk-, and white-RBPs. The numbers of residues in parentheses are from the established sequences. Proteins were hydrolyzed in evacuated sealed tubes with 4 M methane sulfonic acid containing 0.2% 3-(2-amino ethyl)indole for 24, 48, and 72 h at 110°C. Values for serine, threonine, and tryptophan are extrapolated to zero time of hydrolysis. Values for valine and isoleucine are those for 72 h hydrolysis.

| Amino acids and sugars | Plasma-RBP | Yolk-RBP    | White-RBP   |
|------------------------|------------|-------------|-------------|
| Aspartic acid          | 19.1       | 19.6 (19.7) | 19.6 (19.5) |
| Threonine              | 7.0        | 6.8 (7)     | 7.0 (7)     |
| Serine                 | 28.0       | 27.8 (30)   | 28.2 (30)   |
| Glutamic acid          | 33.2       | 28.3 (29)   | 34.5 (35)   |
| Proline                | 8.3        | 8.3 (8)     | 8.1 (8)     |
| Glycine                | 7.2        | 6.6 (6)     | 7.3 (7)     |
| Alanine                | 13.1       | 11.8 (12)   | 13.0 (13)   |
| 1/2 Cystine            | 17.5       | 18.2 (18)   | 17.5 (18)   |
| Valine                 | 5.6        | 5.3 (5)     | 5.2 (5)     |
| Methionine             | 6.5        | 6.8 (7)     | 6.8 (7)     |
| Isoleucine             | 6.5        | 6.5 (7)     | 6.5 (7)     |
| Leucine                | 13.0       | 11.5 (11)   | 13.0 (13)   |
| Tyrosine               | 8.7        | 9.1 (9)     | 8.9 (9)     |
| Phenylalanine          | 5.9        | 4.9 (5)     | 5.9 (6)     |
| Lysine                 | 15.0       | 14.4 (14.3) | 15.2 (15.5) |
| Histidine              | 7.6        | 7.7 (8)     | 7.9 (8)     |
| Tryptophan             | 5.6        | 6.1 (6)     | 5.9 (6)     |
| Arginine               | 5.2        | 5.0 (5)     | 4.9 (5)     |
| Mannose                | 6.0        | 6.0         | 6.0         |
| Galactose              | 4.7        | 4.7         | 1.6         |
| Glucosamine            | 7.5        | 7.8         | 10.5        |
| Sialic acid            | 4.7        | 4.4         | 0.7         |
| Total amino acids      |            | 206         | 219         |

and C-2 were also phosphorylated.

The sequences of CNBr-peptides (B', C-1, D-1, D-2, E, F, H) were determined by the manual Edman method and hydrazinolysis as shown in Fig. 4S. CNBr-peptide B' had the sequence of B linked to G and was derived by incomplete cleavage of a Met-Cys bond during CNBr oxidation. The carboxyl-terminals of peptides C-1 and C-2 were confirmed by the hydrazinolysis method to be Leu and Lys, respectively. Both D-1 and D-2 did not give PTH amino acids at the first step of Edman degradation. After pyroglutamyl aminopeptidase digestion of the peptides, the sequencing could be done. However, longer diges-

tion with the enzyme released a glutamine residue at the second position. A difference between the sequences of D-1 and D-2 was found only at the fourteenth residue, lysine in D-1 and asparagine in D-2, as found in CNBr peptides D-2a and D-2b of white-RBP. This replacement can be explained by one point mutation and the yields of D-1 (27%) and D-2 (68%) reflect the gene frequency in the egg population; that is, the ratio of lysine-RBP and asparagine-RBP was roughly 3 : 7. The sequence of peptide E was determined after removal of phosphates by alkaline phosphatase, since phosphoserine was not detected under our Edman degradation conditions. The dephos-

phorylated peptide was digested with V-8 protease, and three peptides (EV-1-EV-3) were isolated. Their amino acid compositions (Table IIIS) supported the sequence of E as shown in Fig. 4S. The sequence of F was also determined with the aid of V-8 protease (Table IVS).

The longest CNBr-peptide, A, was digested with trypsin and separated into 15 peptides (AT-a-AT-o) by HPLC (Fig. 5S). These peptides were further purified by HPLC with the same buffer system as in Fig. 3S. The amino acid compositions of these tryptic peptides are shown in Table VS. Peptides AT-i, AT-n, and AT-o seemed to be incompletely digested. The amino acid composition of AT-o coincided with the sum of those of AT-1 and AT-m, that of AT-n with the sum of AT-o and AT-b, and that of AT-i with AT-j plus lysine. The sequences of tryptic peptides AT-b-AT-j were determined as shown in Fig. 6S. The complete sequences of AT-k and AT-o were determined by sequence analyses of V8 protease and

chymotrypsin peptides. The sequence of AT-o was the sum of those of AT-m and AT-1 in that order. Since the amino acid sequences of the CNBr-peptides (Fig. 4S) and tryptic peptides of CNBr-peptide A (Fig. 6S) were completely equal to those of white-RBP, except for the C-terminal peptides C-1 or C-2 of yolk-RBP and B of white-RBP (5), the peptides of yolk-RBP were arranged according to the sequence of white-RBP as shown in Fig. 7.

*Elution Patterns of Tryptic Peptides of Yolk-, White-, and Plasma-RCm-RBPs on HPLC*—Tryptic digests of yolk-, white-, and plasma-RCm-RBPs were chromatographed separately under the same HPLC conditions and their elution patterns were compared (Fig. 8).

The elution position of the C-terminal tryptic peptide of white-RBP is shown in the figure by an arrow, since all peaks on the chromatogram of white-RBP had been analyzed in our previous study (see Fig. 9S in our previous paper (5)). The

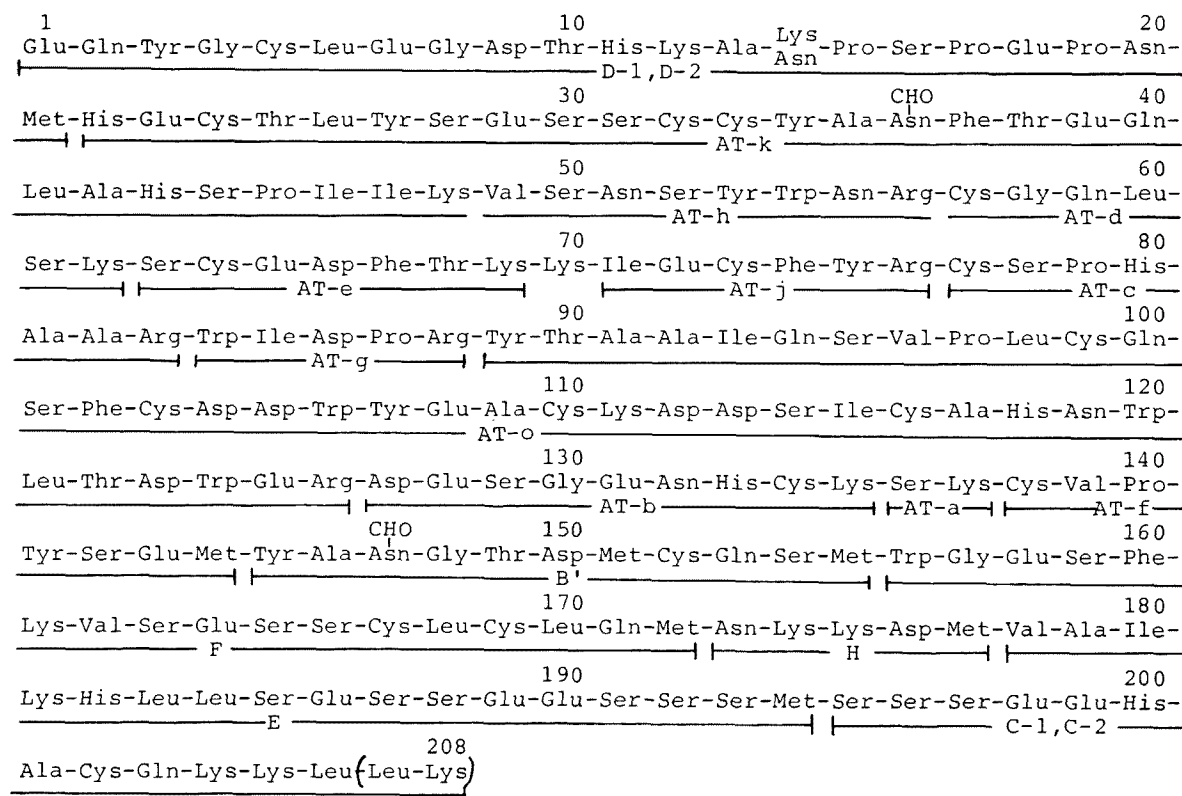


Fig. 7. The complete amino acid sequence of yolk-RBP. About 30% of yolk-RBP had an additional two residues (Leu-Lys) at the C-terminal.

peak of the C-terminal peptide appeared on the chromatogram of the tryptic digest of plasma-RBP, but not on the chromatogram of that of

yolk-RBP. Amino acid analysis and sequence analysis of the peak showed that plasma-RBP had the same carboxyl-terminal sequence as white-RBP.

*Comparison of Amino Acid Compositions of CNBr Peptides of Plasma-, Yolk-, and White-RBPs*—The carboxyl-terminal CNBr-peptide of plasma-RBP was isolated together with other CNBr-peptides by gel filtration on Sephacryl S-200 and HPLC by a similar method to that used for the isolation of CNBr-peptides from white or yolk-RBP. The amino acid compositions of the CNBr-peptides from plasma-RBP are listed in Table VIS. The values are the same as those of the corresponding peptides of white-RBP (5) and also yolk-RBP (Table IIS) except for the carboxyl-terminal CNBr-peptide C in plasma-RBP (Table VIS) and C-1 and C-2 in yolk-RBP (Table IIS). Polymorphism of Lys-RBP and Asn-RBP was also found in plasma-RBP at a similar ratio (3 : 7) to in yolk-RBP, as estimated from the yields of peptides D-1 and D-2 (Table VIS). Peptides A and B were glycosylated and peptides E and C were phosphorylated. The amino acid sequence of peptide C, the C-terminal peptide, was determined as indicated in Fig. 9 with those of yolk- and white-RBPs. The sequence is identical to that of white-RBP but not to that of yolk-RBP.

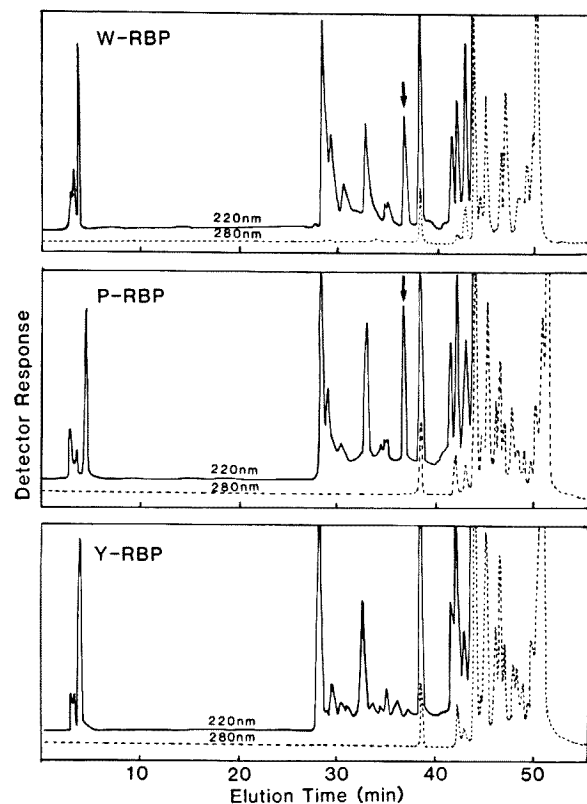
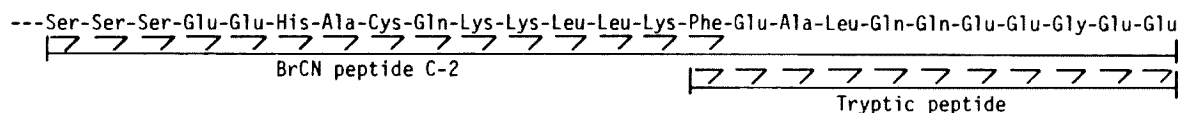


Fig. 8. Comparison of HPLC-patterns of tryptic digests of RCm- white-, yolk-, and plasma-RBPs. The column was of TSK-gel LS-410,  $5\mu$  ( $0.4 \times 30$  cm). Flow rate was 1 ml/min. Chromatography was performed with a linear gradient of 0.01 M ammonium acetate, pH 5.6, and 50% acetonitrile in the buffer.

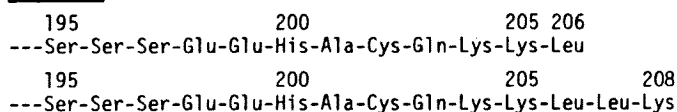
## DISCUSSION

The results of the present study clearly demonstrated that hen plasma-, yolk-, and white-RBPs are the products of the same gene. However,

### Plasma RBP



### Yolk RBP



### White RBP



Fig. 9. Comparison of the sequences of C-terminal CNBr-peptides from plasma-, yolk-, and white-RBPs.

their structures were slightly different due to post-translational modification.

The identity of the amino acid sequence of plasma- and white-RBPs was shown by comparison of the amino acid compositions of CNBr-peptides from plasma-RBP (Table VIS) and white-RBP (Table IIS, in our previous paper (5)), the HPLC patterns (Fig. 8) of tryptic digests of RCm-RBPs and also the results of sequence analysis of the carboxyl-terminal CNBr-peptide of plasma-RBP (Fig. 9). All CNBr-peptides from yolk-RBP had the same amino acid sequences as those of white-RBP, and polymorphism (lysine/asparagine) was observed both in the white- and yolk-RBPs at the fourteenth residue from the N-terminal end.

The carbohydrate compositions (Table I) of the three RBPs showed that the plasma and yolk RBPs had the same sugar compositions, corresponding to those of complex type sugar chains (biantenna or triantenna), and white-RBP had hen egg ovomucoid type sugar chains (8).

The most interesting finding is that the carboxyl-terminal sequence of yolk-RBP is 11 or 13 residues shorter than that of plasma-RBP. The release of the carboxyl-terminal peptide must occur during the incorporation from the blood into the oocyte or during the storage in the yolk fluid. Lipovitellin and phosvitin are known to be produced by the proteolysis of a precursor protein (vitellogenin) which is synthesized in the liver, secreted into the blood and incorporated into the oocyte as well as RBP. Therefore, RBP is the second yolk protein which is enzymatically modified during incorporation or in the yolk fluid after incorporation.

Another interesting processing of RBP is phosphorylation. The phosphorylation sites of white-RBP were several serine residues situated between Ser(185)–Ser(197) (5). The phosphorylation sites of yolk-RBP are probably the same as those of white-RBP, since CNBr-peptides E, C-1, and C-2 are phosphorylated, and have the same structural characteristics; Ser(P)-X-Glu, or Ser(P)-X-Ser(P). This suggested that the same protein kinase or protein kinases with a similar specificity may participate in the phosphorylation of the proteins in both the oviduct and liver. One of our major aims in structural studies on RBPs is the elucidation of the selective uptake of RBP into the oocyte in the ovary. A receptor for RBP is

probably present in the oocyte membrane and plays an important role in the selective uptake like a receptor for vitellogenin or phosvitin found in the oocyte membrane (9).

If there is a receptor for RBP, which part of RBP is important for the binding to the receptor? RBP has some characteristic structures such as a phosphoserine cluster, a C-terminal glutamic acid abundant region and two carbohydrate chains. Although other specific peptide regions can not be excluded as the recognition signal, the above three characteristic regions are strong candidates. Miller *et al.* (10) reported that the removal of a few phosphate residues drastically reduced the uptake of RBP into the oocyte. They also reported that the removal of sialic acid also reduced the rate of incorporation (11, 12). Therefore, the phosphoserine cluster and carbohydrate moiety may be related to the incorporation of RBP.

Usually secreted proteins and some mitochondrial proteins are initially synthesized as preproteins with an NH<sub>2</sub>-terminal pre-segment of 17 to 25 amino acids, and the pre-segment (or signal peptide or leader peptide) is cleaved during passage through the membrane (13–17). Yolk-RBP has a C-terminal sequence that is 11 or 13 residues shorter than that of plasma-RBP. It will be very interesting if the C-terminal peptide of RBP behaves like this pre-segment. However, such a possibility might be ruled out because Miller *et al.* reported that yolk- and plasma-RBPs were incorporated into the oocytes at almost the same rate when both I<sup>125</sup>-labeled RBPs were injected into laying hens (7).

This suggests to us that the C-terminal segment may not be related to the binding with the receptor.

Further study is needed for elucidation of the mechanism of the selective uptake of yolk proteins from the blood into oocyte in the ovary.

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## Supplemental Materials

Table IIS. Amino Acid Compositions of CNBr Peptides of Yolk-RBP<sup>a</sup>

|           | A        | B      | B' <sup>d</sup> | C-1 <sup>c</sup> | C-2 <sup>c</sup> | D-1    | D-2    | E      | F      | G      | H      | total <sup>bc</sup> | Y-RBP <sup>b</sup> |
|-----------|----------|--------|-----------------|------------------|------------------|--------|--------|--------|--------|--------|--------|---------------------|--------------------|
| Cm-Cys    | 13.0(13) |        | 0.9(1)          | 1.0(1)           | 1.0(1)           | 1.0(1) | 0.9(1) |        | 1.6(2) | 0.9(1) |        | 18                  | 16.9(18)           |
| Asp       | 12.8(13) | 2.0(2) | 2.1(2)          |                  |                  | 2.0(2) | 2.6(3) |        |        |        | 2.0(2) | 19.7                | 20.3(19.7)         |
| Thr       | 4.9( 5)  | 1.0(1) | 1.0(1)          |                  |                  | 0.9(1) | 0.8(1) |        |        |        |        | 7                   | 6.3( 7)            |
| Ser       | 13.4(15) |        | 1.0(1)          | 2.4(3)           | 2.5(3)           | 1.0(1) | 1.0(1) | 4.9(6) | 3.8(4) | 1.1(1) |        | 30                  | 28.8(30)           |
| Hse       | 0.7( 1)  | 0.9(1) | 1.5(2)          |                  |                  | 0.5(1) | 0.5(1) | 0.7(1) | 0.7(1) | 0.7(1) | 0.8(1) | 7                   | ( 7)               |
| Glu       | 13.4(14) |        | 0.8(1)          | 3.0(3)           | 3.2(3)           | 3.3(4) | 3.2(4) | 2.8(3) | 2.9(3) | 1.0(1) |        | 28                  | 27.0(28)           |
| Pro       | 5.7( 5)  |        |                 |                  |                  | 3.1(3) | 3.0(3) |        |        |        |        | 8                   | 8.3( 8)            |
| Gly       | 2.3( 2)  | 1.1(1) | 1.1(1)          |                  |                  | 1.9(2) | 1.8(1) |        | 1.2(1) |        |        | 6                   | 6.3( 6)            |
| Ala       | 8.0( 8)  | 1.1(1) | 1.1(1)          | 1.0(1)           | 1.1(1)           | 0.9(1) | 0.9(1) | 1.1(1) |        |        |        | 12                  | 12.5(12)           |
| Val       | 3.0( 3)  |        |                 |                  |                  |        |        | 1.0(1) | 1.0(1) |        |        | 5                   | 5.1( 5)            |
| Ile       | 4.8( 6)  |        |                 |                  |                  |        |        | 0.8(1) |        |        |        | 7                   | 5.2( 7)            |
| Leu       | 5.0( 5)  |        |                 | 1.0(1)           | 2.0(2)           | 1.0(1) | 1.0(1) | 2.0(2) | 1.9(2) |        |        | 11                  | 11.8(11)           |
| Tyr       | 7.0( 7)  | 1.0(1) | 1.0(1)          |                  |                  | 0.9(1) | 0.9(1) |        |        |        |        | 9                   | 9.3( 9)            |
| Phe       | 3.8( 4)  |        |                 |                  |                  |        |        |        | 0.8(1) |        |        | 5                   | 5.0( 5)            |
| Lys       | 7.1( 7)  |        |                 | 2.0(2)           | 3.0(3)           | 1.8(2) | 1.0(1) | 1.3(1) | 1.2(1) |        | 1.8(2) | 14.3                | 14.1(14.3)         |
| His       | 4.9( 5)  |        |                 | 1.0(1)           | 1.0(1)           | 0.9(1) | 0.9(1) | 1.1(1) |        |        |        | 8                   | 8.1( 8)            |
| Trp       | 4.1( 5)  |        |                 |                  |                  |        |        |        | 0.7(1) |        |        | 6                   | 5.7( 6)            |
| Arg       | 4.6( 5)  |        |                 |                  |                  |        |        |        |        |        |        | 5                   | 5.1( 5)            |
| Sugar     | +        | +      | +               |                  |                  |        |        |        |        |        |        | +                   | +                  |
| Phosphate |          |        |                 | +                | +                |        |        | +      |        |        |        | +                   | +                  |
| Total     | 123      | 7      | 11              | 12               | 14               | 21     | 21     | 17     | 17     | 4      | 5      | 206                 | 206                |
| Yield(%)  | 58       | 58     | 7.6             | 28               | 11               | 27     | 68     | 34     | 46     | 17     | 44     |                     |                    |

a, The numbers of residues of yolk-RBP and those in parentheses are from the established sequences.

b, Amino acid replacement in peptides D-1 and D-2 were considered in the calculation.

c, Both C-1 and C-2 were C-terminal CNBr-peptides, and C-1 was used for calculation of the total residues.

d, B' coincides with the sum of B and G.

Table IIIS. Amino Acid Compositions of V8 Protease Peptides of CNBr Peptide E

| Residue  | EV-1   | EV-2   | EV-3   | Total | E      |
|----------|--------|--------|--------|-------|--------|
| Ser      | 1.1(1) | 1.5(2) | 2.0(3) | 6     | 4.5(6) |
| Hse      |        |        | 0.8(1) | 1     | 0.9(1) |
| Glu      | 1.2(1) | 2.0(2) |        | 1     | 2.7(3) |
| Ala      | 1.1(1) |        |        | 1     | 0.9(1) |
| Val      | 1.0(1) |        |        | 1     | 0.8(1) |
| Ile      | 0.9(1) |        |        | 1     | 0.7(1) |
| Leu      | 2.0(2) |        |        | 2     | 2.1(2) |
| Lys      | 1.0(1) |        |        | 1     | 0.9(1) |
| His      | 1.0(1) |        |        | 1     | 0.8(1) |
| Total    | 9      | 4      | 4      | 17    | 17     |
| Yield(%) | 65     | 61     | 52     |       |        |

Table IVS. Amino Acid Compositions of V8 Protease Peptides of CNBr Peptide F

| Residue  | FV-1   | FV-2   | FV-3   | Total | F      |
|----------|--------|--------|--------|-------|--------|
| Cm-Cys   |        |        | 2.1(2) | 2     | 2.1(2) |
| Ser      |        | 1.8(2) | 1.7(2) | 4     | 3.7(4) |
| Hse      |        |        | 0.8(1) | 1     | 0.8(1) |
| Glu      | 1.0(1) | 1.0(1) | 0.8(1) | 3     | 2.9(3) |
| Gly      | 1.0(1) |        |        | 1     | 1.1(1) |
| Val      |        | 1.0(1) |        | 1     | 1.0(1) |
| Leu      |        |        | 2.0(2) | 2     | 2.0(2) |
| Phe      |        | 1.0(1) |        | 1     | 1.1(1) |
| Lys      |        | 1.1(1) |        | 1     | 1.2(1) |
| Trp      | 1.0(1) |        |        | 1     | 1.0(1) |
| Total    | 3      | 6      | 8      | 17    | 17     |
| Yield(%) | 90     | 98     | 82     |       |        |

Table VS. Amino Acid Compositions of Tryptic Peptides of CNBr Peptide A from Yolk-RBP<sup>a</sup>

|          | AT-a   | AT-b   | AT-c   | AT-d   | AT-e   | AT-f   | AT-g   | AT-h   | AT-i   | AT-j <sup>b</sup> | AT-k   | AT-l   | AT-m   | AT-n <sup>b</sup> | AT-o <sup>b</sup> | total <sup>c</sup> |
|----------|--------|--------|--------|--------|--------|--------|--------|--------|--------|-------------------|--------|--------|--------|-------------------|-------------------|--------------------|
| Cm-Cys   | 1.0(1) | 1.0(1) | 1.0(1) | 1.1(1) | 1.0(1) |        |        |        | 0.9(1) | 1.0(1)            | 2.9(3) | 0.9(1) | 2.3(3) | 4.9(5)            | 4.1(4)            | 13                 |
| Asp      | 2.0(2) |        |        | 1.0(1) |        |        | 1.0(1) | 2.0(2) |        |                   | 1.3(1) | 3.5(4) | 1.7(2) | 7.3(8)            | 5.7(6)            | 13                 |
| Thr      |        |        |        | 0.9(1) |        |        |        |        |        |                   | 1.8(2) | 1.0(1) | 0.9(1) | 1.8(2)            | 1.8(2)            | 5                  |
| Ser      | 0.6(1) | 0.9(1) | 1.0(1) | 1.0(1) | 1.0(1) | 0.9(1) |        | 1.9(2) |        |                   | 3.5(4) | 1.2(1) | 2.6(2) | 3.4(4)            | 2.7(3)            | 15                 |
| Hse      |        |        |        |        |        | 1.1(1) |        |        |        |                   |        |        |        |                   |                   | 1                  |
| Glu      | 2.0(2) |        |        | 1.0(1) | 1.0(1) | 0.9(1) |        |        | 1.2(1) | 1.3(1)            | 3.7(4) | 1.3(1) | 2.4(3) | 5.6(6)            | 4.2(4)            | 14                 |
| Pro      |        | 1.0(1) |        |        |        | 0.9(1) | 0.8(1) |        |        |                   | 1.2(1) |        | 1.1(1) | 1.4(1)            | 1.0(1)            | 5                  |
| Gly      | 1.0(1) |        |        | 1.1(1) |        |        |        |        |        |                   |        |        |        | 0.8(1)            |                   | 2                  |
| Ala      |        | 2.0(2) |        |        |        |        |        |        |        |                   | 2.0(2) | 1.0(1) | 1.6(3) | 3.9(4)            | 4.0(4)            | 8                  |
| Val      |        |        |        |        | 1.0(1) |        |        | 0.8(1) |        |                   |        |        | 0.4(1) | 1.0(1)            | 1.0(1)            | 3                  |
| Ile      |        |        |        |        |        |        | 0.8(1) |        | 0.8(1) | 0.9(1)            | 1.1(2) | 0.9(1) | 0.8(1) | 1.9(2)            | 1.9(2)            | 6                  |
| Leu      |        |        | 1.0(1) |        |        |        |        |        |        |                   | 1.8(2) | 1.0(1) | 1.0(1) | 2.1(2)            | 2.0(2)            | 5                  |
| Tyr      |        |        |        |        |        | 1.2(1) |        | 1.0(1) | 0.9(1) | 1.0(1)            | 1.8(2) |        | 1.4(2) | 2.0(2)            | 2.0(2)            | 7                  |
| Phe      |        |        |        | 1.0(1) |        |        |        |        | 0.8(1) | 0.9(1)            | 0.8(1) |        | 0.9(1) | 1.1(1)            | 1.0(1)            | 4                  |
| Lys      | 1.0(1) | 1.0(1) |        | 1.0(1) | 1.0(1) |        |        |        | 0.8(1) |                   |        | 1.0(1) |        | 1.1(1)            | 1.2(1)            | 7                  |
| His      |        | 1.0(1) | 1.0(1) |        |        |        |        |        |        |                   |        | 1.9(2) | 0.8(1) | 1.8(2)            | 1.2(1)            | 5                  |
| Trp      |        |        |        |        |        |        | 0.9(1) | 0.9(1) |        |                   |        | 1.4(2) | 0.5(1) | 3.4(3)            | 2.7(3)            | 5                  |
| Arg      |        | 1.3(1) |        |        |        |        | 0.9(1) | 0.9(1) | 0.8(1) | 0.9(1)            |        | 0.9(1) |        | 0.4(1)            | 0.8(1)            | 5                  |
| Sugar    |        |        |        |        |        |        |        |        |        |                   | +      |        |        |                   |                   | +                  |
| Total    | 2      | 9      | 7      | 6      | 7      | 7      | 5      | 8      | 7      | 6                 | 27     | 15     | 23     | 47                | 38                | 123                |
| Yield(%) | 65     | 43     | 68     | 63     | 49     | 67     | 79     | 67     | 13     | 47                | 55     | 11     | 23     | 17                | 50                |                    |

a, The numbers in parentheses are from the established sequences.

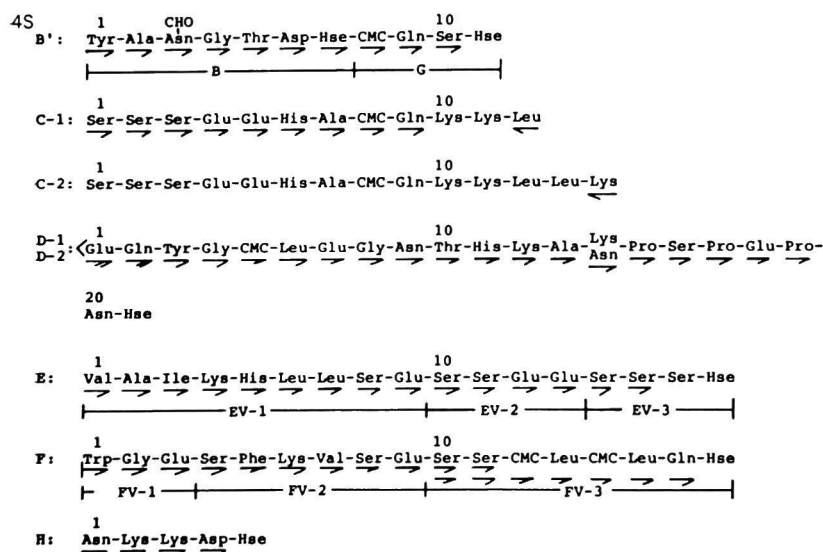
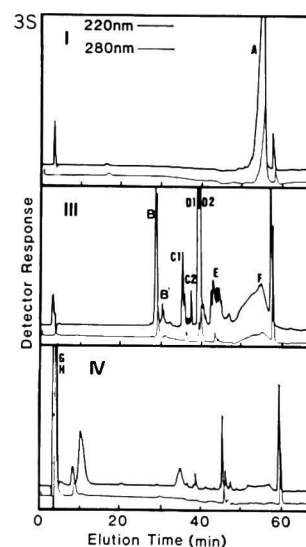
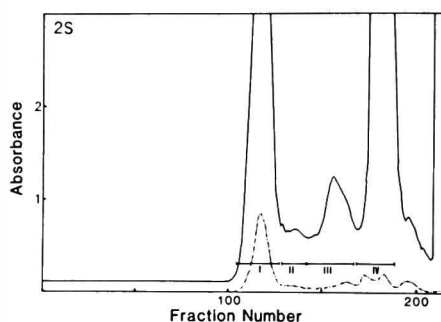
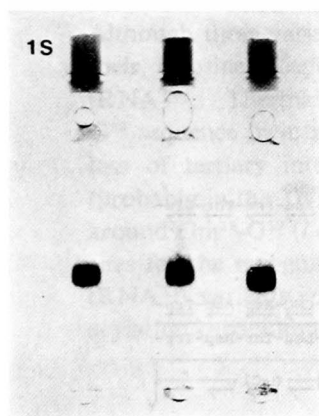
b, Incompletely hydrolyzed peptides: AT-i=(AT-j)+Lys; AT-o=(AT-l)+(AT-m); AT-n=(AT-l)+(AT-m)+(AT-b).

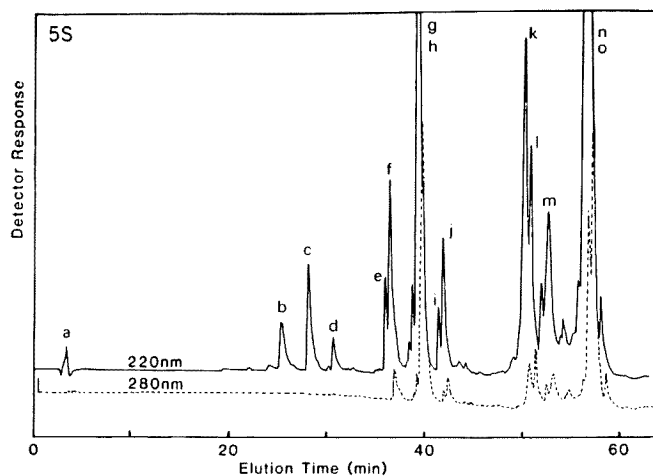
c, AT-j, AT-m and AT-o are excluded.

Table VIS. Amino Acid Compositions of CNBr Peptides of Plasma-RBP<sup>a</sup>

|           | A (A)    | B (D-1) | C (B)   | D-1 (D-2a) | D-2 (D-2b) | E (E)  | F (C)  | G (F-1) | H (F-2) | Total <sup>b</sup> |
|-----------|----------|---------|---------|------------|------------|--------|--------|---------|---------|--------------------|
| Cm-Cys    | 13.0(13) |         | 1.1(1)  | 0.8(1)     | 0.8(1)     | 2.0(2) |        | 0.8(1)  |         | 18                 |
| Asp       | 13.4(13) | 2.0(2)  |         | 1.9(2)     | 2.5(3)     |        |        |         | 2.1(2)  | 19.7               |
| Thr       | 4.5(5)   | 1.0(1)  |         | 0.9(1)     | 0.9(1)     |        |        |         |         | 7                  |
| Ser       | 14.1(15) |         | 2.4(3)  | 0.7(1)     | 0.7(1)     | 3.8(4) | 4.5(6) | 1.0(1)  |         | 30                 |
| Hse       | 0.9(1)   | 1.0(1)  |         | 0.7(1)     | 0.7(1)     | 0.9(1) | 1.0(1) | 0.9(1)  | 0.9(1)  | 7                  |
| Glu       | 13.9(14) |         | 9.8(10) | 3.5(4)     | 3.7(4)     | 2.8(3) | 2.9(3) | 1.0(1)  |         | 35                 |
| Pro       | 4.5(5)   |         |         | 2.2(3)     | 2.1(3)     |        |        |         |         | 8                  |
| Gly       | 2.3(2)   | 1.0(1)  | 1.2(1)  | 2.3(2)     | 2.3(2)     | 1.0(1) |        |         |         | 7                  |
| Ala       | 8.1(8)   | 1.0(1)  | 2.0(2)  | 1.1(1)     | 1.2(1)     |        | 1.0(1) |         |         | 13                 |
| Val       | 3.1(3)   |         |         |            |            | 1.0(1) | 1.2(1) |         |         | 5                  |
| Ile       | 4.9(6)   |         |         |            |            |        | 1.1(1) |         |         | 7                  |
| Leu       | 5.0(5)   |         | 3.0(3)  | 0.9(1)     | 0.9(1)     | 1.9(2) | 1.9(2) |         |         | 13                 |
| Tyr       | 6.6(7)   | 1.0(1)  |         | 0.8(1)     | 0.9(1)     |        |        |         |         | 9                  |
| Phe       | 3.8(4)   |         | 1.0(1)  |            |            | 0.9(1) |        |         |         | 6                  |
| Lys       | 6.9(7)   |         | 3.0(3)  | 1.5(2)     | 0.9(1)     | 1.1(1) | 0.9(1) |         | 2.0(2)  | 15.3               |
| His       | 4.5(5)   |         | 1.1(1)  | 0.9(1)     | 0.9(1)     |        | 0.9(1) |         |         | 8                  |
| Trp       | 4.7(5)   |         |         |            |            |        |        |         |         | 5                  |
| Arg       | 4.5(5)   |         |         |            |            |        |        |         |         | 5                  |
| Total     | 123      | 7       | 25      | 21         | 21         | 17     | 17     | 4       | 5       | 219                |
| Sugar     | +        | +       |         |            |            |        |        |         |         | +                  |
| Phosphate |          |         | +       |            |            | +      |        |         |         | +                  |
| Yield(%)  | 42       | 79      | 41      | 28         | 62         | 68     | 36     | 22      | 32      |                    |

a, The numbers of residues of Plasma-RBP and those in parentheses were evaluated on the basis of the corresponding peptides of white-RBP. The peptide names in parentheses are the names of corresponding white-RBP peptides designated in our previous paper in Table IIS(5).  
b, Amino acid replacement in peptides D-1 and D-2 was considered in the calculation.





6S a: Ser-Lys

b: Asp-Glu-Ser-Gly-Glu-Asn-His-CMC-Lys

c: CMC-Ser-Pro-His-Ala-Ala-Arg

d: CMC-Gly-Gln-Leu-Ser-Lys

e: Ser-CMC-Glu-Asp-Phe-Thr-Lys

f: CMC-Val-Pro-Tyr-Ser-Glu-Hse

g: Trp-Ile-Asp-Pro-Arg

h: Val-Ser-Asn-Ser-Tyr-Trp-Asn-Arg

i: Lys-Ile-Glu-CMC-Phe-Tyr-Arg

j: Ile-Glu-CMC-Phe-Tyr-Arg

k: His-Glu-CMC-Thr-Leu-Tyr-Ser-Glu-Ser-Ser-CMC-CMC-Tyr-Ala-Asn-Phe-Thr-Glu-  
Gln-Leu-Ala-His-Ser-Pro-Ile-Ile-Lys + h + d + e

o: Tyr-Thr-Ala-Ala-Ile-Gln-Ser-Val-Pro-Leu-CMC-Gln-Ser-Phe-CMC-Asp-Asp-Trp-  
Tyr-Glu-Ala-CMC-Lys-Asp-Asp-Ser-Ile-CMC-Ala-His-Asn-Trp-Leu-Thr-Asp-Trp-  
Glu-Arg  
O-C2

Fig. 1S. Polyacrylamide gel electrophoresis of plasma-RBP(1), yolk-RBP(2) and white-RBP(3). Electrophoresis was carried out using 7% acrylamide gel at pH 8.0 according to Williams and Reisfeld (18).

Fig. 2S. Gel filtration of RCM-Yolk-RBP cleaved by CNBr. The column was a Sephacryl S-200 column (2.0 x 200 cm). The solvent was 0.02M  $\text{NH}_4\text{HCO}_3$  (pH 8.0). The fraction size was 3 ml. Peptides were monitored at 220nm (—) and 280 nm (---).

Fig. 3S. HPLC of fractions I, III and IV in Fig. 2S. The column was of TSK-Gel LS-410, 5μ (0.4 x 30 cm). Chromatography was performed with a 60 min linear gradient at a flow rate of 1 ml/min using a primary solvent of 0.05% TFA and a secondary solvent system of 2-propanol-acetonitrile-0.05%TFA(42:18:40, v/v/v). Peptides were monitored at 220 nm and 280 nm.

Fig. 4S. The amino acid sequences of CNBr peptides of Yolk-RBP. CMC represents carboxymethyl cysteine;  $\langle$  Glu, pyroglutamyl residue; CHO, carbohydrate chain;  $\rightarrow$ , manual Edman degradation;  $\leftarrow$ , hydrazinolysis;  $\Rightarrow$ , pyroglutamyl amino-peptidase. Prefix V indicates V8 protease peptides.

Fig. 5S. Separation of tryptic peptides of CNBr peptide A by HPLC. The conditions were the same in Fig. 3S except for the solvents. The primary solvent was 0.01 M ammonium acetate buffer, pH 5.6, and the secondary solvent was 50% acetonitrile in 0.01 M ammonium acetate, pH 5.6.

Fig. 6S. The amino acid sequences of tryptic peptides of CNBr peptide A determined by manual Edman degradation.  $\rightarrow$ , manual Edman degradation. O-C1 and O-C2 were chymotryptic peptides. A-V was a peptide obtained by V8-protease digestion of CNBr peptide A, which included the C-terminal end 9 amino acids of k and peptides h, d and e.