Note

Preparation of Amino-Terminal Half-Molecule of Ovotransferrin by Tryptic Digestion of Intact Molecule Selectively Saturated with Al(III) at the N-Lobe

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Ovotransferrin (OTf) is composed of a single glycosylated polypeptide with the molecular weight of 80,000. The amino- and carboxyl-terminal halves of the chain are homologous in their sequences and folded into similar compact lobes. Each lobe contains a binding site for a metal ion. The two lobes of apo-OTf can be rapidly digested into small inactive peptides by proteases and are stabilized against proteases by metal binding. Therefore, one simple method for the preparation of half-molecules of OTf is selective incorporation of a metal ion into the N- or C-lobe and then digestion of the apo-lobe by protease. For selective incorporation of Fe(III) into the N-lobe, iron nitrotriacetate has been used as an iron donor at slightly alkaline pH. Unfortunately, much Fe₃OTf remained after tryptic digestion, suggesting that the C-lobe is also occupied by Fe(III), presumably because of cooperation of the two sites in Fe(III) binding. This gives a low yield of the N-terminal half-molecule and contaminates the C-terminal half-molecule, the iron-complex of which is more resistant than the N-terminal half-molecule incorporated in such digestion. Thus, if preparation of pure N-terminal half-molecules is to be made possible, a new method for the stabilization of the N-lobe is needed. Ichimura et al. reported that the N-lobe of OTf binds more readily than the other lobe with Al(III), and that the two lobes compete during Al-binding. Therefore, when a small amount of Al(III) is mixed with apo-OTf, OTf in which only the N-lobe is bound with Al(III) should be obtained.

We report here a simple method for the preparation of N-terminal half-molecules of OTf by the selective tryptic digestion of C-terminal apo-lobes of OTf partly saturated with Al(III).

Ovotransferrin (OTf) was prepared from chicken egg white as described previously. Metal-free ovotransferrin (apo-OTf) was dissolved in 0.1 M Tris-HCl buffer containing 10 mM CaCl₂ and 2 mM NaHCO₃ at pH 8.0. The apo-OTf solution was digested with trypsin (Sigma; 0.5 mg/ml) for 3 or 7 hr at 25°C and pH 8.0. The hydrolysate was stopped by the addition of 10% trichloroacetic acid (TCA) and the sample was used for SDS-PAGE on 10% acrylamide gel. The protein bands were stained with Coomassie Brilliant Blue R-250.

Figure 1 shows SDS-PAGE patterns of apo-OTf partly saturated with Al and Al₃OTf, all treated with trypsin for 3 hr at 25°C. Most of the apo-OTf molecules were digested into small peptides that were not detected by SDS-PAGE used (lane 2). The binding of apo-OTf with Al somewhat inhibited tryptic digestion. Two large fragments in bands A and B (lane 8) with the apparent molecular mass of about 40 kDa were produced from Al₃OTf. The mobilities of the two fragments were consistent with those of N- and C-terminal half-molecules derived from Fe₃OTf treated with trypsin (lane 9). The fragments of bands A and B (from Al₃OTf and Fe₃OTf) respectively were electrophoresed onto polyvinylidene difluoride membranes and their sequences were found to be AlaProProProLysSerValle and GlnAsnArgHis, respectively, with a pulse-labeled sequence. These sequences were consistent with the N-terminal sequences reported for N- and C-terminal half-molecules of OTf derived from Fe₃OTf treated with trypsin. Thus, the bands A and B were N- and C-terminal half-molecules of OTf, respectively.

Band B increased as the Al-saturation of OTf increased (lanes 3—8), but band A remained almost constant. When OTf saturated 50% with Al was treated with trypsin (lane 3), band B was faint. This indicated that the N-lobe of OTf was selectively saturated with Al(III), and that the Al-bound N-lobe was resistant to tryptic digestion. The SDS-PAGE patterns of tryptic hydrolysates of OTf partly saturated with Al (lanes 3—8) also showed that the stabilizing effect of Al-binding with the C-lobe was less than that of binding with the N-lobe, as the staining intensity of band B was lower than that of band A even when 80—100% Al-bound OTf was used (lanes 6—8).

Much undigested OTf and a trace of C-terminal half-molecules remained after the tryptic digestion of 50% Al-saturated OTf for 3 hr (lane 3). Therefore, for high-yield preparation of N-terminal half-molecules without digestion by C-lobe fragments, OTf saturated 45% with Al was treated with trypsin for 7 hr. Figure 2 shows the elution pattern of the hydrolysate passed through a Sephadex G-100 column. The first peak corresponded to N-terminal half-molecules of OTf, and the second peak corresponded to small inactive peptides. Uncleaved 80-kDa OTf was not observed (arrow in Fig. 2). The N-terminal half-molecules obtained after gel filtration (lane 3) gave a single band on SDS-PAGE (inset), and the mobility of the fragment was consistent with that of the N-terminal half-molecules derived from the tryptic digestion of Fe₃OTf (upper band, lane 2). A single band also appeared on SDS-PAGE after the reduction of the N-terminal half-molecule with 2-mercaptoethanol, indicating that the fragment was a single polypeptide. The yield of N-terminal half-molecules was 38 mg from 125 mg of OTf.

Abbreviations: OTf, ovotransferrin; apo-OTf, metal-free ovotransferrin; Al₃OTf, aluminum-saturated ovotransferrin; Fe₃OTf, ferric ovotransferrin; and SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
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10 Fig. 1. Protective Effects of Al Complexes with OTf against Tryptic Digestion.

Apo-OTf, OTf partly saturated with Al, OTf saturated with Al were treated with trypsin for 3 hr and the hydrolysates were analyzed by SDS-PAGE. Lane 1, OTf before trypsin treatment; lane 2, apo-OTf; lanes 3, 4, 5, 6, and 7 contained OTf saturated 50, 60, 70, 80, or 90%, respectively, with Al; lane 8, Al₃OTf; lane 9, a mixture of N- and C-terminal half-molecules derived from trypsin-nicked Fe₃OTf. Disulfide bridges in the proteins were not reduced. For details, see the text.
OTF saturated 45% with Al (25 mg/ml) was treated with trypsin (0.5 mg/ml) for 7 h at 25°C (pH 8.0). The hydrolysis reaction was stopped by the addition of soybean trypsin inhibitor (0.7 mg/ml), and 5.0 ml of the mixture was passed through a Sephadex G-100 column (2.0 x 90 cm) equilibrated with 50 mM NH4HCO3. The column was eluted with the same solution, 2.0-ml fractions of the eluted solution was collected, and fractions containing half-molecules of OTF (indicated by the horizontal bar) were pooled. The arrow shows the elution position expected for intact OTF. The inset shows SDS-PAGE of intact OTF (Lane 1), a mixture of N- and C-terminal half-molecules from trypsin-nicked Fe3OTF (lane 2), and fragments obtained after the gel filtration (lane 3).

The OTF molecule is composed of N- and C-lobes each of about 40 kDa, so the yield of N-terminal half-molecules was about 60%. Spectroscopic titration of the N-terminal half-molecules obtained here with Fe(III)-nitrilotriacetate and Cu(III) indicated maximum binding of 0.98 mol of iron/mole of protein and 0.93 mol of copper/mole of protein. The max of the visible absorption spectrum of Cu-bound N-terminal half-molecules was 450 nm, which was the same as that of Cu-bound N-terminal lobes of intact OTF. This indicated that the N-terminal half-molecules retained an intact micro-environment around the metal-binding site of the N-terminal lobe of intact OTF.

The method that we report here is simpler than the methods reported previously, which include isolation of monoferric species from partially iron-saturated OTF with an electofocusing column and then long-time incubation of the monoferric OTF with trypsin, or else the preparation of trypsin-nicked OTF and then the separation of N- and C-terminal half-molecules by cation-exchange chromatography. The yield of N-terminal half-molecules by our method was about 60%, compared to the 20–30% of other methods.

Iron-bound C-lobes of OTF are more resistant to trypsin than iron-bound N-lobes. The stabilizing effect of Al-binding to the N-lobes was greater than the effect of Al-binding to the C-lobe. This, together with selective Al incorporation into N-lobe, permitted high-yield preparation of N-terminal half-molecules of OTF without contamination by C-terminal half-molecules.

References