# Dimer of S-100 Proteins

Sadayuki MATSUDA
Hokkaido University of Education at Asahikawa, 9, Hokumoncho, Asahikawa, Hokkaido 070
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**Synopsis.** The association of the S-100 protein was studied by the HPLC of gel filtration in the presence of 0.5 mM $Zn^{2+}$. To avoid any association of the proteins, it was necessary for the dissoving buffer to contain urea of 6 M concentration. This conclusion was confirmed by fluorescence measurements of S-100a.a′.

S-100 proteins are $Ca^{2+}$-binding proteins which exist mainly in the brain as a mixture of three isoforms (S-100a, S-100a′ and S-100b) with subunits of $\alpha\beta$, $\alpha'\beta$, and $\beta\beta$, respectively.[1—3] Namely, S-100a.a′ and S-100b were heterodimer and homodimer, respectively. It is known that each isoform has the same molecular weight (21000).[3] The characteristic properties of the S-100a and S-100a′ are very similar, and those of the S-100b are different from the former.[4] It is also known that S-100 proteins bind with $Zn^{2+}$ more tightly than to $Ca^{2+}$; this binding is significant physiologically.[5]

On the basis of Sephacryl S-200 gel chromatography, Kay et al. have reported that the dimer of the S-100 protein was obtained if the concentration of the protein in the dissolvation was as low as 1—4 mg ml$^{-1}$.[6—8] On the other hand, the presence of oligomers other than the dimer has been found by a small-angle X-ray scattering (SAXS) technique or HPLC of the gel filtration method.[9—11] It is indispensable in studying this protein to prepare a sample having the same degree of association, or possibly the dimer alone. It has been reported that the presence of trifluoperazine (TFP) and EDTA is necessary to obtain the dimer alone.[9—11]

In this study it has been shown that the existence of urea of 6 M concentration (1 M=1 mol dm$^{-3}$) in the dissolvation buffer has a decisive effect for practically preparing the dimer alone. This new method has the advantage that the addition of TFP is unnecessary, even in the presence of $Zn^{2+}$. At the same protein concentration, a distinct difference in the fluorescence intensity was observed between the dimer alone and the mixture of the dimer and other oligomers.

## Experimental

**Materials.** S-100a.a′ and S-100b were prepared as previously reported.[11] The powder of freeze-dried S-100a.a′ and S-100b was treated under two different dissolvation conditions, one with urea and the other without urea. The buffer has the following composition: 20 mM 3-morpholino-1-propanesulfonic acid (MOPS)–NaOH (pH=6.6) and 2 mM 2-mercaptoethanol in the presence or absence of 6 M urea. The dissolvation was followed by dialyzing to the same buffer twice; urea was then removed by dialysis while changing the solvent (without urea) at least 4 times.

**Methods.** The HPLC of gel filtration was carried out at room temperature using a Waters Protein Pak-125 column (7.8×300 mm). The running buffer was 0.2 M $(NH_4)_2SO_4$, 20 mM MOPS–NaOH (pH=6.6), 0.5 mM $ZnSO_4$ and 2 mM 2-mercaptoethanol, and the flow rate was 0.5 ml min$^{-1}$. The absorbance at 280 nm was monitored.

Fluorescence measurements were obtained with a Hitachi MPF-4 spectrofluorometer at room temperature.

## Results and Discussion

Figure 1 shows the elution curves of S-100a.a′ and S-100b in the presence of 0.5 mM $ZnSO_4$. In the case of the conventional dissolvation method (no urea), S-100a.a′ was eluted in three peaks (Fig. 1A); this was also the case for S-100b (Fig. 1C), as already reported.[11] Two peaks (the first and second peaks in Figs. 1A and 1C) correspond to the oligomers. The third large peak is assigned to the dimer based on the molecular weight (21000) of S-100 protein.[11] The calibration curve under these conditions for a molecular weight estimation is shown in Fig. 2. Although S-100a.a′ and S-100b have the same molecular weight, the retention times of these proteins differ slightly. This is because they have a slightly different molecular shape.[9,10] A similar elution curve with Figs. 1A and 1C was observed in the preparation of the precipitate obtained by saturated $(NH_4)_2SO_4$ at pH=4.2. This is also the case for the precipitate obtained by trichloroacetic acid. These aggregations were clearly observed at a concentration of 1.1 mg ml$^{-1}$, which was the range of the concentration for the dimer noted by Kay et al.[6—8] Although the retention time did not depend on the protein concentration, the ratio of aggregates to dimer slightly increased with the protein concentration. On the contrary, both S-100a.a′ and S-100b, prepared using 6 M urea, were eluted in only one peak, which corresponds to the dimer (Figs. 1B and 1D). Although the protein concentration of Figs. 1B and 1D (4.2 mg ml$^{-1}$) was 4-times that of Figs. 1A and 1C, aggregation did not occur at all. Since 6—8 M urea is usually usd in isolation of troponin C from troponin,[12] the possibility of the denaturation of S-100 proteins seems to be negligible. The characteristics of $Zn^{2+}$ and $Ca^{2+}$ binding of S-100a.a′ and S-100b did not change at all by a 6 M urea treatment. That the only dimer species could be obtained with a 6 M urea treatment was also confirmed by the SAXS technique (N. Matsushima, S. Matsuda, and Y. Izumi, unpublished results).

Since Kay et al. prepared their S-100 proteins using the conventional method (no urea), their preparation

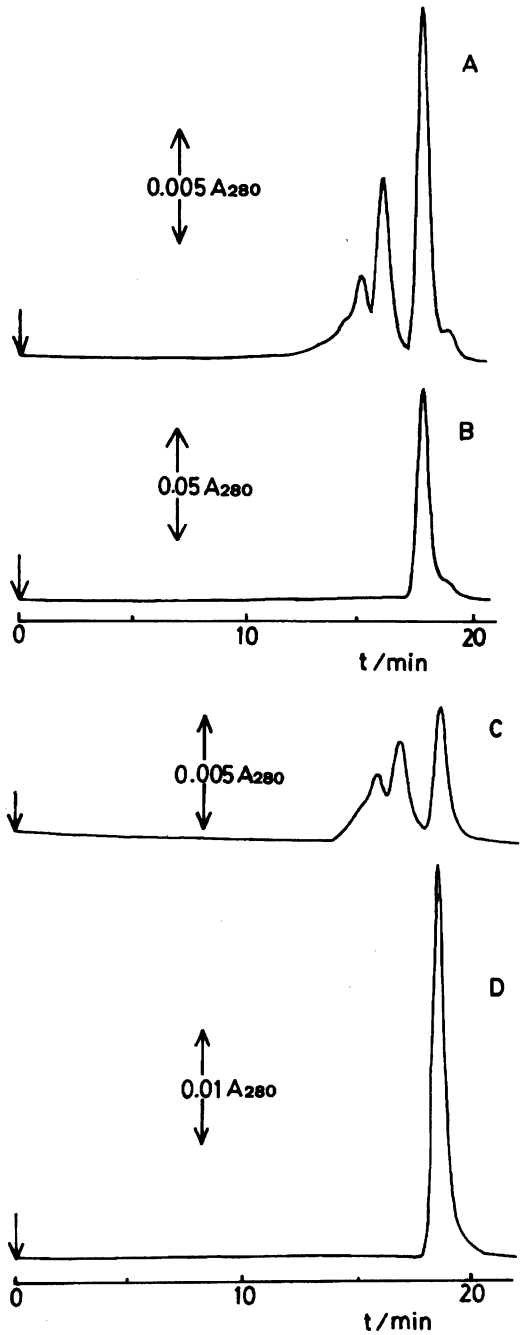


Fig. 1. HPLC of S-100a.a′ (A, B) and S-100b (C, D) on a Protein Pak-125 column in the presence of 0.5 mM $ZnSO_4$. Each sample was injected at the time indicated by an arrow. The injected volume and concentration of each sample (A, B, C, and D) were 15 μl and 1.1 mg ml$^{-1}$, 15 μl and 4.2 mg ml$^{-1}$, 30 μl and 1.1 mg ml$^{-1}$, and 15 μl and 4.2 mg ml$^{-1}$, respectively. Samples A and C were prepared with the conventional dissolvation of freeze-dried powder. Samples B and D were prepared with the dissolvation freeze-dried powder in a buffer containing 6 M urea.

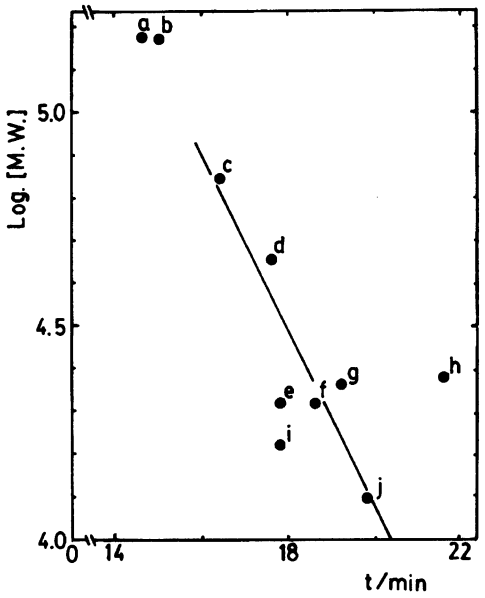


Fig. 2. Calibration curve for the dimers of S-100a.a′ and S-100b obtained by a Protein Pak-125 column in the presence of 0.2 M $(NH_4)_2SO_4$, 20 mM MOPS–NaOH, and 0.5 mM $ZnSO_4$. The retention times of standard proteins were plotted versus the logarithm of their molecular weight: (a), bovine γ-globulin; (b), alcohol dehydrogenase (yeast); (c), bovine serum albumin; (d), egg albumin; (e), S-100a.a′; (f), S-100b; (g), chymotrypsinogen A; (h), trypsin inhibitor (soybean); (i), bovine brain calmodulin; (J), cytochrome c (horse heart).

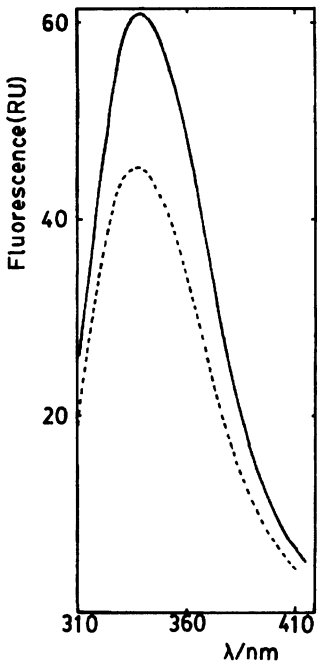


Fig. 3. Fluorescence spectra of 0.8 mg ml$^{-1}$ S-100a.a′ in the presence of 0.1 M NaCl, 20 mM MOSP–NaOH, 0.5 mM $ZnSO_4$, and 2 mM 2-mercaptoethanol. The solid line and dashed line denote the spectrum of sample A and sample B in Fig. 1, respectively. S-100a.a′ was excited at 290 nm.

might contain some oligomers other than a dimer.[6—8] They supposed as a dimer their S-100 preparation because the elution pattern showed a single peak on Sephacryl S-200 open-column chromatography.[7] For precision, the use of open-column chromatography is insufficient compared with HPLC.

Figure 3 shows the fluorescence spectra of S-100a.a′ in the presence of 0.5 mM $Zn^{2+}$. It is found that the fluorescence intensity of S-100a.a′ obtained with a 6 M urea treatment was 74% of that of S-100a.a′ obtained by the conventional method for the same protein concentration. The different fluorescence spectra are attributed to the difference in the composition. Namely, one is a mixture of oligomers and the dimer; the other is the dimer alone. Since the hydrophobicity around the tryptophan residue of the dimer is less than that of the oligomers, the fluorescence intensity of S-100a.a′ prepared using 6 M urea is less than that of S-100a.a′ obtained without urea. The result of Fig. 3 is reasonably derived from the result of Fig. 1.

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