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The role of *N*-acetyl-methioninate as a new stabilizer for albumin products

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Abstract

Sodium octanoate (Oct) and *N*-acetyl-L-tryptophanate (*N*-AcTrp) are widely used as stabilizers during the pasteurization of albumin products. However, *N*-AcTrp has a possible side effect of intracerebral disease. To provide safe and risk-free albumin products, we validated *N*-acetyl-methioninate (*N*-AcMet) as a new stabilizer for albumin products. The effect of *N*-AcMet on oxidation was examined using 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH) as an oxidizing agent. Carbonyl content in the presence of *N*-AcMet, as well as that in the presence of *N*-AcTrp after 24 h (Anraku et al., 2004), was significantly decreased. The effect of AAPH on the oxidative status of 34-Cys on human serum albumin was also studied by HPLC. It was found that *N*-AcMet as well as *N*-AcTrp, has a large protective effect on the sulfhydryl group after 1 h. Further, *N*-AcMet was found to be a superior radical scavenger to *N*-AcTrp using 1,1′-diphenyl-2-picrylhydrazyl (DPPH) radicals. The thermal stabilizing role of *N*-AcMet manifested as an increase in denaturation temperature and calorimetric enthalpy, as determined by differential scanning calorimetry (DSC). In the present study, we suggest that use of *N*-AcMet in albumin preparation is safe and free of risk of side effects.

Keywords: Albumin; Sulfhydryl groups; Calorimetry (DSC); Antioxidant activity; *N*-Acetyl-L-methioninate; Oxidation

1. Introduction

Human serum albumin (HSA) is the most abundant protein in plasma, and in addition to being the primary colloid, it serves as an important transport and depot protein (Peters, 1996). Large amounts of albumin are used clinically during surgery and to treat shock trauma. As the only source of HSA for clinical application is donated human blood, the risk of transmitting pathogenic viruses, such as those causing hepatitis, HIV, and as yet unidentified diseases, exists. Pasteurization of HSA is carried out by heating at 60 °C for several hours with sodium octanoate (Oct) and *N*-acetyl-L-tryptophanate (*N*-AcTrp) as commonly used stabilizers (Shrake et al., 1984; Ross et al., 1984), a process that usually destroys the viruses present. These commonly used additives effectively protect HSA by increasing the melting temperature as determined by differential scanning calorimetry (DSC) and decreasing the formation of aggregates after heating (Arakawa and Kita, 2000). We have previously shown that Oct has the greatest stabilizing effect against heat, while *N*-AcTrp diminishes oxidation of HSA (Anraku et al., 2004). However, *N*-AcTrp has a possible side effect of intracerebral disease (Aguilera et al., 2001). In Trp metabolism, 3-hydroxykynurenine is known to have particularly strong neurotoxic properties (Topczewska-Bruns et al., 2003), and the accumulation of Trp metabolites in nervous tissue due to HSA product administration may be involved in pathogenesis of several neurological disorders in uremia. To provide safe and risk-free albumin preparations, it is important to find new stabilizing reagents instead of *N*-AcTrp.

All amino acid residues of proteins are susceptible to oxidative modification by one or more forms of reactive oxygen species (ROS) (Vogt, 1995; Brot and Weissbach, 1983). The oxidative modifications of sulfur-containing amino acids such as cysteine and methionine (Met) could serve as antioxidants via their cyclic oxidation and reduction. In particular, Met residues of proteins are susceptible to oxidation by almost all forms of ROS (Vogt, 1995). On the other hand, no effect on oxidation was found for *N*-acetyl-cysteinate in our previous studies, although cysteine (Cys) residues of proteins are susceptible to oxidation (Anraku et al., 2004). Thus, we focused on a sulfur-containing

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amino acid having mercapto groups, *N*-acetyl-methioninate (*N*-AcMet).

In the present study, we investigated the protective effect of *N*-AcMet on the oxidation of albumin. In addition, we investigated the stabilizing effect of *N*-AcMet by differential scanning calorimetry (DSC). We suggest that the co-use of *N*-AcMet and Oct produces an excellent stabilizing effect on albumin and depresses agglomeration safely and without any risk of side effects.

2. Materials and methods

2.1. Materials

HSA donated by Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and genetically recombinant human serum albumin (rHSA) donated by Nipro Corporation (Shiga, Japan) were defatted using charcoal treatment as described by Chen (1967). After dialysis against distilled water, the protein fraction was freeze-dried and stored at −20 °C until use. While HSA was used for all the experiments, rHSA was used only for the expected examination for the clinical application. *N*-Acetyl-L-methioninate (*N*-AcMet) and *N*-acetyl-L-tryptophanate (*N*-AcTrp), were purchased from Nacalai Tesque (Kyoto, Japan). Fluoresceinamine (isomer II) and sodium octanoate (Oct) were purchased from Sigma Chemical Co. (St. Louis, MO). 2,2′-Azobis(2-amidino-propane)dihydrochloride (AAPH) and 1,1′-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Methods

2.2.1. Effect of oxidation on HSA in the presence and absence of ligands

HSA (50 μM), with and without (250 μM) additives, was oxidized by exposure to AAPH (10 mM) in 67 mM sodium phosphate buffer (pH 7.4, 37 °C), as described by Niki (1987). After incubation for 1 or 24 h, oxidation was stopped by the addition of acetone. Protein carbonyl content was determined using the method of Climent et al. (1989). The carbonyl groups were derivatized with fluoresceinamine and their numbers were calculated from the absorbancy of the complexes at 490 nm (Jasco Ubest-35 UV–vis spectrophotometer). Mercaptalbumin (HMA; reduced form) and nonmercaptalbumin (HNA-1 and -2; oxidized forms) were separated by application to an HPLC-column packed with *N*-methylpyridinium polymer cross-linked with ethylene glycol dimethacrylate, prepared as described previously (Sugii et al., 1989; Narazaki et al., 1997). From the HPLC profiles of HSA, the values of each albumin fractions (f(HMA), f(HNA-1), and f(HNA-2)) were estimated by dividing the area of each fraction by the total area corresponding to HSA.

2.2.2. Scavenging of DPPH (1,1′-diphenyl-2-picrylhydrazyl) radicals in solution

Radical scavenging activity of *N*-AcMet and *N*-AcTrp was tested in ethanolic solution (10 ml of ethanol, 10 ml of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5) and 5 ml of 0.5 mM DPPH in ethanol) with an albumin concentration of 50 μM and a ligand concentration of 250 μM. Radical scavenging was estimated from the decrease in absorbance of DPPH radicals at 517 nm due to scavenging of an unpaired electron from stable DPPH radicals by ligands (Kogure et al., 1999).

2.2.3. Effect of heating on HSA in the presence and absence of ligands

Differential scanning calorimetry (DSC) was carried out on HSA with a protein concentration of 0.1 mM in 67 mM sodium phosphate buffer, pH 7.4 using a MicroCal MC-2 ultrasensitive DSC (MicroCal Inc., Northampton, MA) with a heating rate of 1 K/min. The calorimetric reversibility of the thermally induced transition was checked by reheating the cooled protein solution from the first run in the calorimetric cell, which was flushed with nitrogen. The results showed, as was also observed by Picó (1997), that heating to or above 85 °C causes irreversible denaturation. The data obtained from DSC were applied to non-linear fitting algorithms to calculate thermodynamic parameters of thermal denaturation temperature (T_m), calorimetric enthalpy (ΔH_{cal}) and van't Hoff enthalpy (ΔH_v), and analyzed by Using Origin™ scientific plotting software to determine C_p from the temperature dependence of excess molar heat capacity. Each sample was recorded before heating and after heating to 60 °C for 30 min.

2.2.4. Thermal stabilities on HSAs in the presence and absence of N-AcMet for clinical application

Aqueous solutions (25%, w/v) were prepared by dissolving HSA and rHSA in 500 ml physiological saline. Then, stabilizers Oct (1662 mg) and *N*-AcMet (1912.5 mg) were added and dissolved, and 50-ml aliquots of HSA or rHSA solutions were hermetically sealed in 50-ml vials. The samples were subjected to heat treatment under pasteurization conditions of 60 °C for 30 min, and the generation of contaminants was observed.

2.2.5. Statistics

Statistical significance was evaluated using ANOVA followed by the Newman–Keuls method for comparisons of more than two means. A value of $p < 0.05$ was regarded as statistically significant. Results are reported as mean ± S.D.

3. Results

3.1. Effect of oxidation on HSA in the presence and absence of ligands

HSA exposed to AAPH results in the formation of carbonyl groups. The carbonyl content of HSA, which has not been exposed to AAPH, was 0.037 ± 0.002 mol/mol protein for all the samples ($n = 3$). It is evident from Fig. 1 that the carbonylation increased with incubation time. The presence of Oct has no inhibiting effect on carbonyl formation after 1 hr exposure to AAPH, while *N*-AcMet had a protective effect against prolonged exposure to the oxidant. Similar results were also observed for

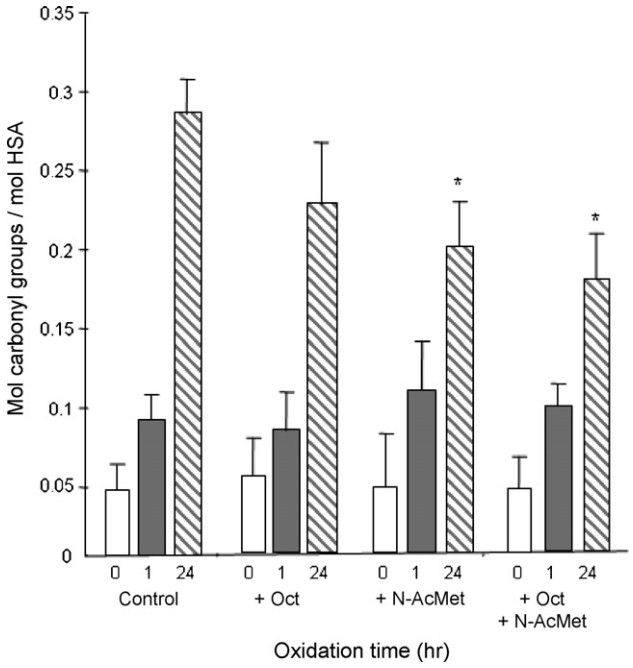

Fig. 1. AAPH-induced oxidation of HSA alone (control) and in the presence of ligands. The values are mean ± S.D. ($n = 4$). * $p < 0.01$ as compared with control.

N-AcTrp, reported previously (Anraku et al., 2004). Protection against oxidation was even more pronounced when both *N*-AcMet and Oct were present; this effect was mainly due to the presence of *N*-AcMet because addition of Oct alone gave no significant protection (Fig. 1).

We also investigated whether AAPH treatment for 1 h affects the status of the SH group of 34-Cys using quantitative analysis of chromatograms given in Table 1 and Fig. 2. Exposure to AAPH resulted in a pronounced reduction in the amount of HMA, the relative area of the peak decreased from 65.1% to 22.3%, showing that AAPH was also capable of oxidizing SH groups of Cys residues. This hypothesis was supported by a calculation of peak areas (Table 1). N-AcMet had a protective effect in the presence or absence of Oct. Quantitative analysis presented in Table 1 shows that this effect was significant for *N*-AcMet, as well as for *N*-AcTrp (Anraku et al., 2004).

Table 1
Relative fractions of HMA, HNA-1 and HNA-2 (%) before and after oxidation byAAPH[a]

	HSA alone	+Oct	+*N*-AcMet	+Oct + *N*-AcMet
Before oxidation				
HMA	65.1 ± 2.3	66.1 ± 2.9	65.3 ± 2.5	65.3 ± 2.5
HNA-1	22.8 ± 2.5	23.4 ± 4.2	21.5 ± 3.5	22.5 ± 3.7
HNA-2	10.6 ± 5.3	10.8 ± 4.5	11.6 ± 4.5	11.5 ± 5.4
After oxidation				
HMA	22.3 ± 2.8	22.3 ± 2.5	39.3 ± 2.3*	39.2 ± 2.6*
HNA-1	51.4 ± 2.5	51.3 ± 4.2	46.3 ± 3.2*	45.6 ± 3.4*
HNA-2	26.7 ± 5.3	26.5 ± 4.5	12.4 ± 4.5*	15.5 ± 5.5*

[a] The concentration of HSA was 50 μM, and that of AAPH and the ligands was 10 mM and 250 μM, respectively. The results are average values ± S.D. for three experiments.
* $p < 0.05$ as compared with HSA alone.

3.2. Scavenging of DPPH radicals in solution

The effects of *N*-AcMet and *N*-AcTrp were examined by monitoring the change in the absorbance of DPPH radicals during 15-min incubation. As shown in Fig. 3, HSA with *N*-AcMet

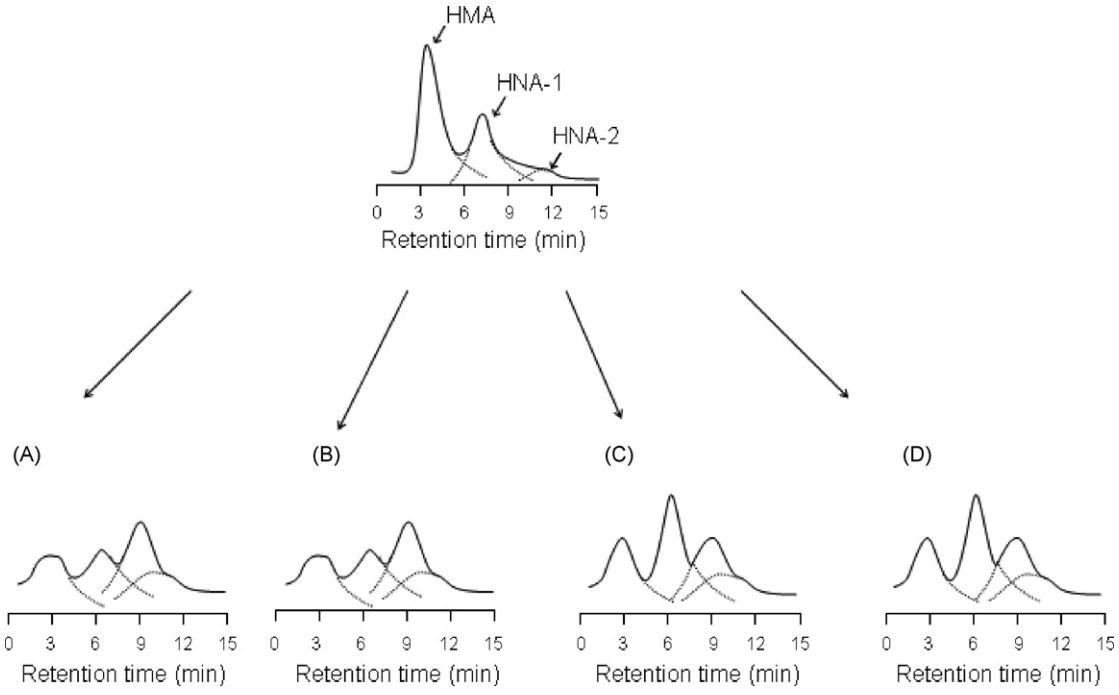

Fig. 2. HPLC chromotograms of HSA before (upper curves) and after AAPH-induced oxidation (lower curves). (A) Oxidation of albumin in absence of additives, (B) oxidation in the presence of Oct, (C) oxidation in the presence of *N*-AcMet, (D) oxidation in the presence of *N*-AcMet and Oct. Peaks correspond to HMA, HNA-1 and HNA-2. Data shown are from representative experiments.

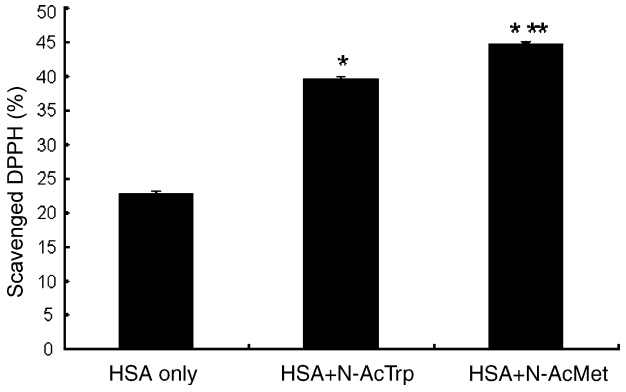

Fig. 3. Radical scavenging ability of HSA with ligands. $^{*}p<0.05$, compared with blank. $^{**}p<0.05$, compared with *N*-AcTrp.

and *N*-AcTrp were better scavengers of DPPH radicals than HSA alone. It should be noted that the scavenging ability of *N*-AcMet was higher than that of *N*-AcTrp (*N*-AcTrp; 39.5 ± 0.3, *N*-AcMet; 44.6 ± 0.4, $p<0.05$).

3.3. Effect of heating on HSA in the presence and absence of N-AcMet

The effect of *N*-AcMet on the thermogram of HSA as determined by DSC was examined. For HSA, which had not been preheated, the addition of the ligands shifts the thermogram towards higher temperature as follows (Fig. 4A): Oct + *N*-AcMet > Oct > *N*-AcMet > HSA alone. The results obtained after preheating these samples are shown in Fig. 4B. In this case, no normal thermogram was obtained for HSA alone. However, addition of additives protected albumin during preheating, and these samples resulted in thermograms which were shifted towards higher temperatures in the same as in Fig. 4A. As shown in Table 2, the addition of *N*-AcMet alone produced a slight increase in T_m and an increase in ΔH_{cal} before and after preheating. Further, incubation of HSA with Oct and *N*-AcMet produced a more stable state than HSA alone. The effect of *N*-AcMet was similar to that of *N*-AcTrp (Anraku et al., 2004).

Table 2
Thermodynamic data obtained from DSC of different HSA samples at pH 7.4[a]

Protein samples	T_m (°C)	ΔH_{cal} ($\times 10^2$ kcal/mol)	$\Delta H_v/\Delta H_{cal}$ ($\times 10^2$ kcal/mol)
Without preheating			
HSA	59.50 ± 0.06	1.64 ± 0.11	0.7 ± 0.01
HSA+Oct	66.83 ± 2.10	2.10 ± 0.15	0.7 ± 0.01
HSA + *N*-AcMet	60.06 ± 0.10	1.71 ± 0.05	0.7 ± 0.03
HSA + Oct + *N*-AcMet	66.51 ± 0.15	2.03 ± 0.09	0.7 ± 0.01
With preheating[b]			
HSA	ND[c]	ND	ND
HSA + Oct	66.80 ± 0.04	2.10 ± 0.19	0.7 ± 0.01
HSA + *N*-AcMet	61.58 ± 0.06	0.91 ± 0.25	1.9 ± 0.01
HSA + Oct + *N*-AcMet	66.46 ± 0.05	1.96 ± 0.25	0.8 ± 0.02

[a] The concentration of HSA was 0.1 mM, and that of the ligands was 0.5 mM. The results are average values ± S.D. for three experiments.
[b] Preheating was incubation at 60 °C for 30 min.
[c] No normal thermogram could be detected.

3.4. Thermal stabilities of HSAs in the presence and absence of N-AcMet for clinical application

As a result, in the sample without the addition of the additive, the aggregate of HSA and rHSA by heating were distinctly generated. On the other hand, no contaminant was observed in the two samples with the addition of *N*-AcMet (data not shown).

4. Discussion

For hypoalbuminemia, which is hard to control, albumin preparations are used for supplementation to improve clinical conditions. Specifically, albumin is indispensable for modern medical treatment because they are generally used for collection of a circulating plasma volume in hemorrhagoc and traumatic shock, improvement of edema, and various kinds of diseases such as liver cirrhosis and nephrotic syndrome (Peters, 1996).

Conventionally, the production of HSA involves fractionating blood collected from human sources, and purifying the obtained albumin-containing aqueous solution according to var-

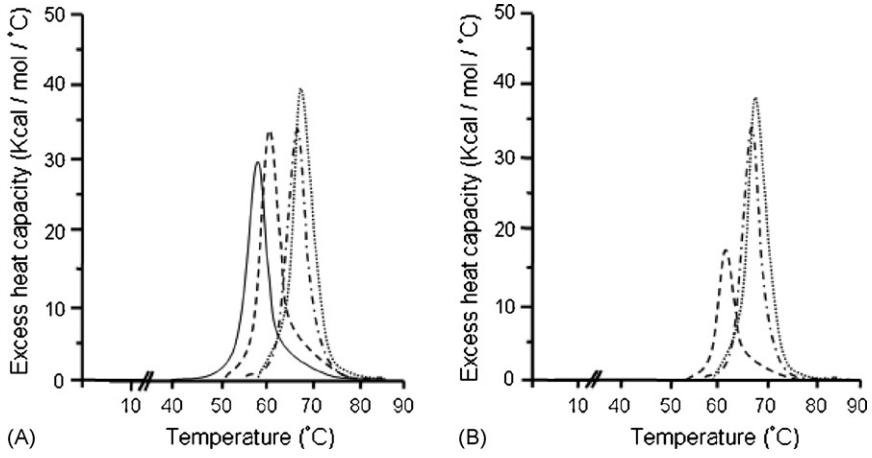

Fig. 4. Effect of Oct and *N*-AcMet on the thermogram of HSA obtained by DSC. (A) Curves for samples that were not preheated. (B) Curves for samples which have been preheated for 30 min at 60 °C. Results are shown for HSA alone (—), HSA with *N*-AcMet (- - -), HSA with Oct (- · - · -), and HSA with both ligands (· · ·). Data are averages of three experiments.

ious purification methods. In the production of HSA, low-temperature pasteurization (60 °C for 10 h) is performed to remove harmful heat-sensitive viruses and to prevent protein contamination is performed. Because the viruses are usually destroyed by heating at 60 °C for several hours without denature of HSA. In low-temperature pasteurization, *N*-AcTrp and Oct are added to HSA as stabilizers (Ballou et al., 1944; Boyer et al., 1946), but *N*-AcTrp has a possible side effect of intracerebral disease (Aguilera et al., 2001). The resulting accumulation of Trp metabolites in nervous tissue may be involved in pathogenesis of several neurological disorders in uremia (Topczewska-Bruns et al., 2003). To provide HSA preparations safely and without risk of side effects, it is important to find new stabilizing reagents instead of *N*-AcTrp.

Moskovitz et al. (1997) recently suggested that oxidation of surface-exposed Met residues to Met sulfoxide is a defense against extensive and irreversible oxidative modification of proteins. Further, Bourdon et al. (2005) suggested that Cys primarily acts as a free radical scavenger, whereas Met primarily acts as a metal chelator on the HSA molecule, although other amino acid residues are likely to be involved in the anti-/prooxidant properties of HSA. Under such circumstances, we focused on *N*-AcMet.

In the present studies, we first compared the protective effect of *N*-AcMet with *N*-AcTrp on oxidation of HSA. HSA was exposed to AAPH, which oxidizes histidine, Trp, tyrosine and Met residues in proteins (Ma et al., 1999; Bourdon et al., 2005). As shown in Fig. 1, the carbonyl content of HSA increased with incubation time. By contrast, *N*-AcMet had a protective effect during prolonged exposure to oxidant, as does *N*-AcTrp (Anraku et al., 2004). In mercaptalbumin (HMA) the last Cys residue, at position 34, has a free SH group. *In vivo*, a major part of the group is either bound to free Cys or glutathione (HNA-1) or is oxidized to sulfenic, sulfinic or sulfonic states (HNA-2). In this study, we also investigated whether AAPH-treatment for 1 hr affects the status of the SH group of 34-Cys. Exposure to AAPH resulted in oxidization of SH-groups of Cys residues (Fig. 2 and Table 1). Incubating Oct together with AAPH has no effect on oxidation. By contrast, the presence of *N*-AcMet has a protective effect, which was the same for the absence (Fig. 2C) or presence (Fig. 2D) of Oct. Thus, with respect to the potential oxidation of HSA, *N*-AcMet, but not Oct, had a significant protective effect. In addition, we found by using DPPH radical that *N*-AcMet produced significant protection against free radicals, compared to *N*-AcTrp (Fig. 3). This suggests that *N*-AcMet is more useful than *N*-AcTrp as a stabilizer.

To validate the benefit of *N*-AcMet as a stabilizer, we also studied the stabilizing effect of *N*-AcMet by DSC. It is seen that the endotherms were single and sharp peaks, indicating that thermal denaturation can be explained by a single component model (Picó, 1997; Anraku et al., 2001). Therefore, single values for T_m, ΔH_{cal} and ΔH_v could be calculated (Table 2). Further, ΔH_{cal}, which is generally thought to reflect the hydration of hydrophobic regions buried in the native protein structure during the unfolding process, varied similarly to the T_m values. The ratio of $\Delta H_v/\Delta H_{cal}$ is an index of the transition process to the denaturation states of proteins during thermal denaturation (Kosa et al., 1998). The values of these ratios for preheated samples with Oct were almost identical to those for unheated samples, whereas those of the *N*-AcMet containing sample increased. Thus, Oct gave a greater protection against heat stress than *N*-AcMet. In our previous work, we have also studied in some detail the effect of *N*-AcTrp on the structural stabilities of HSA. Experiments performed with circular dichronism and native-PAGE showed that Oct and *N*-AcTrp have a pronounced stabilizing effect on the structure of monomeric HSA during heating, as shown by higher T_m- and ΔH_{cal}-values (Anraku et al., 2004). In the case of *N*-AcMet and Oct, we also observed structural stabilities of HSA for these experiments (data not shown).

In recent years, technology for the mass production of albumin with recombinant HSA has been established (Storch, 1993). In the near future, highly stable and inexpensive rHSA stabilized by *N*-AcMet may be available for clinical application.

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