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Heat-treatment method for producing fatty acid-bound alpha-lactalbumin that induces tumor cell death

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ABSTRACT

HAMLET (Human Alpha-lactalbumin Made LEthal to Tumor cells), which was identified in human breast milk as an alpha-lactalbumin (LA)–oleic acid complex, kills tumor cells, selectively. Although it may have potential as a therapeutic agent against various tumor cells, only low-volume methods for its production exist. In this study, heat treatment was used to produce complexes from LAs and oleic acid using a simple method. In the case of human LA and oleic acid, heat-treated samples apparently showed much stronger activities than those treated at room temperature, with cytotoxicities equal to that of HAMLET. Furthermore, circular dichroism spectroscopy revealed that heat-treated samples lost their tertiary structure, suggesting a molten globule as oleic acid-bound LA. BLA samples also showed strong activities by heat treatment. Batch production with heat treatment can efficiently convert LAs into tumoricidal complexes.

Alpha-lactalbumin (LA) is a small globular protein secreted in mammalian milks. It is a calcium-binding metalloprotein, which becomes a stable molten globule (MG) under low-pH, Ca-free and low-salt conditions, under thermal stress or in the presence of mild denaturants. A great number of studies have been performed to investigate the characteristics of the intermediate folding and metal-binding affinity of LA [1–4].

In the lactating mammary gland, alpha-lactalbumin takes a part in the components of lactose synthase [5]. Meanwhile, it has been reported that LA and its derivative from human milk have the ability to induce apoptosis-like cell death in tumor cells. Moreover, Svanborg and coworkers have identified that a complex of apo-HLA and oleic acid (HAMLET; Human Alpha-lactalbumin Made LEthal to Tumor cells) induces apoptosis, or programmed cell death, in tumor cells but spares mature cells [6–10]. Interestingly, these complexes adopt an MG-like state under physiological conditions at neutral pH [11,12].

For the efficient *in vitro* formulation of such complexes (HAMLET), only one method has been demonstrated: Apo-LA was bound on an anion-exchange column in which oleic acid had been loaded and then the fraction eluted with high salt was collected as the active complex. It was also noted that a simple mixing experiment with apo-LA and oleic acid showed much lower activity than HAMLET [6,13].

Purified bovine LA or the LA of other species, together with oleic acid can be converted to HAMLET-like complexes by the same method [14,15]. Although it would be of benefit to prepare the active components from such milk as LAs of domestic animals are more abundant and convenient resources for clinical or industrial application, the low yields of the active fraction using an anion-exchange column pose a problem.

In this study, we prepared a complex directly from a mixture of LA and oleic acid for the purpose of practical and effective conversion of functional materials. We found that heat treatment induces partial denaturation for LA without a calcium chelator and converts LA into the complexes efficiently. The CD spectrum clearly suggested that the heat-treated mixture maintains an MG-like state stably even after cooling and the removal of free oleic acid. This complex induces tumor cell death as efficiently as HAMLET, i.e., the complex from an anion-exchange column. In addition, our method produced cytotoxic active complexes from BLA more easily than the conventional method.

Abbreviations: LA, alpha-lactalbumin; MG, molten globule; HAMLET, human alpha-lactalbumin made lethal to tumor cells; CD, circular dichroism; HLA, human alpha-lactalbumin; BLA, bovine alpha-lactalbumin; FCS, fetal calf serum; PBS, phosphate-buffered saline; UV, ultraviolet.

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Materials and methods

Bovine and human alpha-lactalbumin (BLA and HLA). Holo-alpha-lactalbumin from bovine and human milk, oleic acid, RPMI-1640, fetal calf serum, non-essential amino acids, gentamicin, and sodium pyruvate were purchased from Sigma. All other reagents were of biochemical research grade.

Heat treatment of alpha-lactalbumin–oleic acid mixture. Alpha-lactalbumins were dissolved at 210 μM in phosphate-buffered saline (PBS). Oleic acids were directly suspended into the LA solutions for 120 molar equivalents. These mixtures were heated (50 °C and 60 °C) to facilitate dispersal of the oleic acid and structural change of the protein. After incubation for 10 min, the mixtures were cooled to room temperature. Excess oleic acids were carefully removed by centrifugation.

Culture of L1210. Mouse lymphocytic leukemic cell line L1210 was obtained from the JCRB cell bank. The cells were maintained in RPMI-1640 supplemented with non-essential amino acids, gentamicin, sodium pyruvate, and 10% fetal calf serum at 37 °C in an atmosphere of 95% humidity and 5% carbon dioxide.

Viability of L1210 cells. L1210 cells were washed by PBS and re-suspended at a density of 2×10^6 cells/ml in RPMI-1640 media without FCS. The cell culture was seeded in a 96-well plate. The tested compounds were added to cell cultures to final concentrations of 21 μM (LA conc.). After incubation for 30 min at 37 °C in 5% CO_2, FCS was added to the culture. After 6 h incubation, cell viabilities were evaluated by MTS colorimetric assay (CellTiter 96 Aqueous non-radioactive proliferation assay, Promega) according to the manufacturer's instructions.

Agarose gel electrophoresis of DNA fragmentation. Untreated cells (control) and drug-treated cells (2×10^6/ml) were harvested and washed twice with ice-cold PBS. The cell pellets were re-suspended in lysis buffer (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100) and left to stand for 10 min on ice. The suspension was then centrifuged at 16,000*g* for 5 min. The supernatant was treated with DNase-free RNase A for 60 min at 37 °C and with Proteinase K for 30 min at 50 °C. NaCl was added to the supernatant to a final concentration of 0.2 M, and nucleic acids were precipitated by addition of 2-propanol and overnight incubation at −20 °C. After centrifugation, the entire pellets were dissolved in 20 μl of TE buffer (10 mM Tris, 10 mM EDTA, pH 8.0). The DNA samples were run on 2% agarose gel electrophoresis, visualized by ethidium bromide staining and photographed under UV light.

CD spectroscopy. CD spectra were measured with a Jasco J-725A spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The pass length of the optical cuvette was 10 mm for 320–250 nm and 1 mm for 250–205 nm, respectively. The protein concentrations were 21 μM for all measurements. The temperature of the measuring cells was maintained at 37 °C. The protein concentrations were determined by the absorbance at 280 nm according to Gill and von Hippel [16].

Results and discussion

Cell-death activity of heat-treated HLA–oleic acid

This experiment was performed to establish a convenient method to convert LA and fatty acid into active tumoricidal complexes. Fig. 1 shows the cytotoxicities of our samples against L1210 leukemia cells. Excess oleic acid or LAs themselves possessed almost no cytotoxicity (Fig. 1, Nos. 2, 3, and 7). A purified HAMLET sample, produced by the conventional method using an anion-exchange column, was used as the control. The simple mixture of HLA and oleic acid showed slight cytotoxicity but was much lower than that of HAMLET (Fig. 1, Nos. 1 and 4).

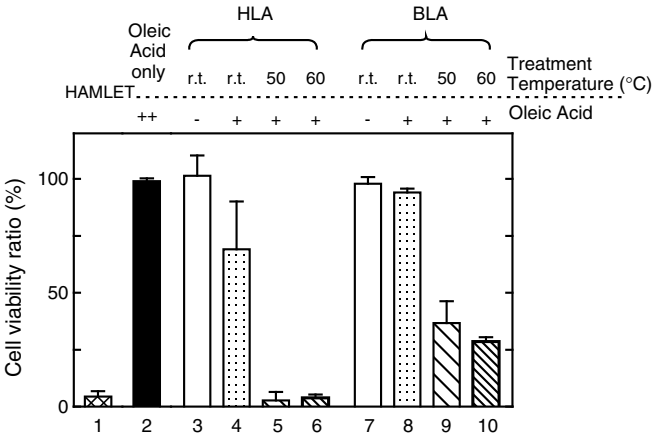

Fig. 1. Viabilities of L1210 cells after 6 h exposure with 21 μM HAMLET (No. 1), 2.5 mM oleic acid (No. 2), 21 μM HLA (No. 3), 21 μM HLA samples treated at room temperature, 50 °C and 60 °C (Nos. 4, 5, and 6), 21 μM BLA (No. 7), and 21 μM BLA samples treated at room temperature, 50 °C and 60 °C (Nos. 8, 9, and 10). Results are presented as means ± SD ($n = 5$).

HAMLET has been thought to adopt partially unfolded states under physiological conditions [6]. It has been reported that the structural features of HAMLET are very similar to those of the intermediate states of LA during denaturation [17,18]. To examine whether denaturation of LA would cause an increase in the bioactive component, we attempted heat-induced denaturation of HLA in the presence of oleic acid. Briefly, authentic HLA was dissolved in PBS. About one hundred molar equivalents of oleic acid were directly suspended in the solution. The solution was heated for a fixed time and then cooled. Excess fatty acid was subsequently removed. Intriguingly, heat-treated samples showed a stronger activity compared with the simple mixture (Fig. 1, Nos. 5 and 6). The mean values of the viability ratio were 3% for the heat-treated samples at 50 °C and 4% at 60 °C. These activities were comparable to that of HAMLET.

Further confirmation of the activity of heat-treated samples was performed by DNA ladder assays. Fig. 2 shows the results of the electrophoresis of DNA isolated from L1210 cells. DNA fragmentation, which is one of the characteristic features of apoptosis, was observed in both HAMLET and the heat-treated samples (Fig. 2, lanes 2 and 3). These results clearly suggested that the cytotoxic function of the heat-treated samples was similar to that of HAMLET.

To confirm the characteristics of the structure of the heat-treated samples, CD spectra were measured at pH 7.4 and 37 °C (Fig. 3). The near-UV CD spectrum of HLA had the characteristics of a negative peak at 270 nm and a positive peak at 293 nm, indicating the rigid packing interactions of aromatic side-chains (Fig. 3A). Heat-treated samples and HAMLET had lower signal intensities compared to native LA, indicating the loss of this tertiary structure. The far-UV CD spectra of both native HLA and heat-treated complexes showed significant negative ellipticities (Fig. 3A inset). CD ellipticity at 222 nm is commonly used as a measure of the extent of secondary structures. Thus, it seemed that the spectrum of the heat-treated complex showed an increase in helicity from that of native LA. These spectroscopic features of the samples agreed with those of the molten globule state of LA [19]. Interestingly, HAMLET showed weak negative peaks in the near-UV CD spectrum, which indicates that HAMLET partially maintains the native-like tertiary structure. On the other hand, the tertiary structures of heat-treated complexes were thought to be completely lost from their CD spectra. This result may indicate that structural features of heat-treated complexes are slightly different from that of HAMLET although their apoptotic activities resemble each other.

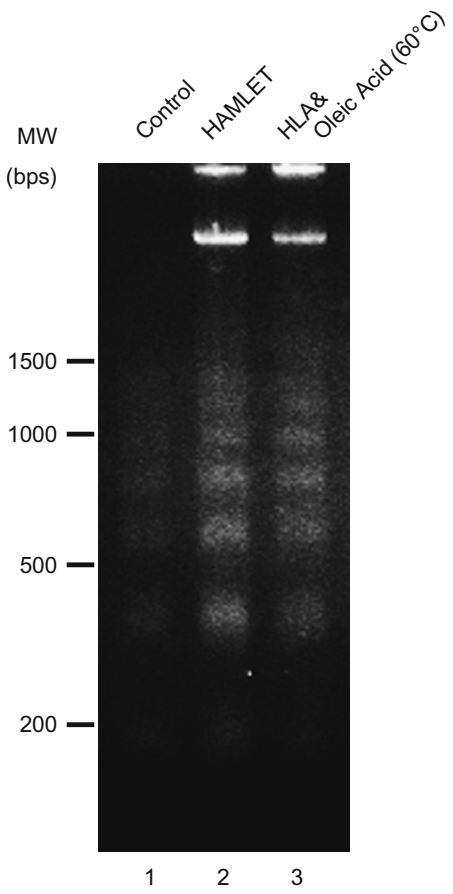

Fig. 2. DNA fragmentation of L1210 cells. Cell culture with control (lane 1), HAMLET (lane 2), and HLA samples treated at 60 °C (lane 3) after 3 h exposure to each sample.

Oleic acid is thought to be hardly bound to holo-LA because calcium binding increased the structural stability of native LA at room temperature. By contrast, heat-treated samples showed a conformational transition toward the intermediate state that suggested the binding of oleic acid; even calcium was not depleted from LA. LA can be partially unfolded, and Ca^{2+} will still remain associated with the protein [20]. Thus, heat treatment of LA would facilitate the exposure of hydrophobic residues to water and binding to oleic acid regardless of the presence of calcium. In terms of practical efficiency, the batch process with heat treatment in this study seemed more suitable for producing active components than the conventional method, the scale and condition of which are limited by the anion-exchange resin.

Cell-death activity of heat-treated BLA–oleic acid

It has been reported that conversion of BLA into the apoptotic complex using an anion-exchange column results in low yields compared to HLA [15,16]. Hence, we tried to produce the apoptotic complex using the batch process with heat treatment. A simple mixture of BLA and oleic acid had almost no cell-death activity in contrast to the above case of HLA and oleic acid (Fig. 1, No. 8). On the other hand, heat-treated complexes apparently increased the apoptotic activities (Fig. 1, Nos. 9 and 10). The cell viabilities of BLA–oleic acid complexes were 37% for the heat-treated sample at 50 °C and 29% at 60 °C, respectively. BLA clearly formed apoptotic complexes, although the activities were lower than those of HAMLET or heat-treated HLA complexes.

The CD spectra of BLA samples showed slight differences from those of HLA samples. Heat-treated samples of BLA showed a weak negative peak around 270 nm in near-UV CD spectra although the intensity was much lower than the native state (Fig. 3B). Thus, the heat-treated BLA–oleic acid complex may partially maintain its native-like tertiary structure. This residual peak at around 270 nm of the 50 °C-treated sample was diminished by 60 °C treatment. Interestingly, the peak intensities of the near-UV seemed to be cor-

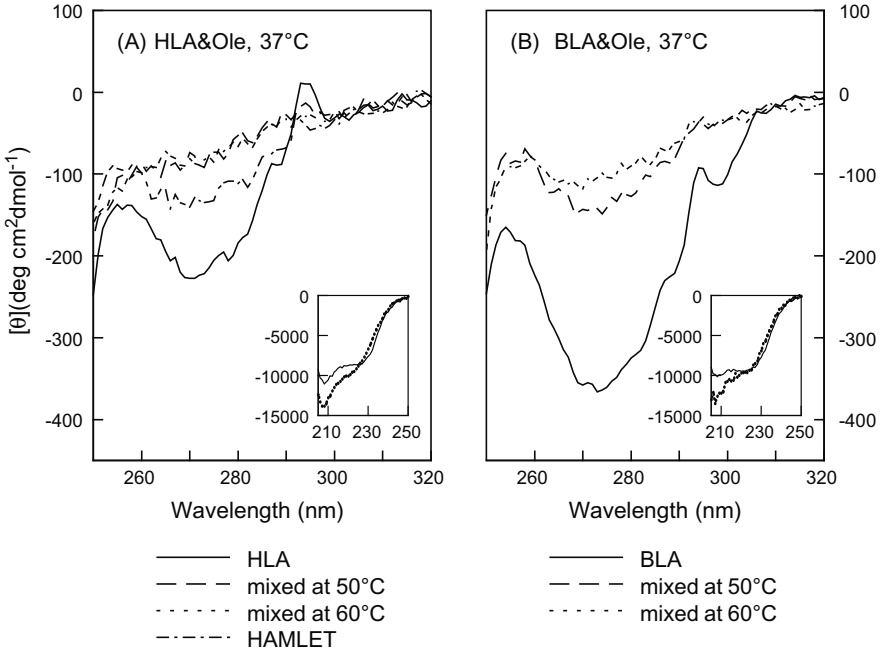

Fig. 3. Effects of heat treatment on both (A) HLA–oleic acid and (B) BLA–oleic acid samples represented by CD spectra in the near-UV region. All spectra were measured at 37 °C for native LA (solid), heat-treated samples at 50 °C (dash) and 60 °C (dot), and HAMLET (dash–dot). Each sample was dissolved in PBS (pH 7.4). Inset shows the far-UV region of native LA (solid) and heat-treated samples at 60 °C (dot).

related with cytotoxic activity. These results may indicate that the amount of the active components increases as native LA loses its conformation by heat treatment. However, heat treatment at 70 °C or 80 °C was less effective for apoptotic activity and peak intensities of near-UV CD spectra (data not shown). These results may imply that an unnecessarily high temperature interrupts the hydrophobic association between LA and oleic acid and prevents the formation of the active components. Studies on LA folding have indicated that the stability of the MG state of HLA is substantially greater than that of BLA [2,21–25]. Thus, HLA can adopt the MG state, which is thought to be important for forming active components, more easily than BLA. One advantage of our batch method is that reaction conditions are easily controlled. There is a possibility that other appropriate conditions of denaturation would improve the productivity of the active components of BLA.

It was reported that the calcium-depleted form of LA and its loading on an anion-exchange column preconditioned with oleic acid were essential for converting LA and oleic acid to a complex showing strong activity against tumor cells. However, heat treatment changed such a mixture to a cytotoxic component without a calcium chelator. Utilizing the batch method, the conversion of BLA to an apoptotic complex was successful. This method could be capable of effective conversion of the active components from LAs.

Acknowledgments

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References

[1] K. Kuwajima, The molten globule state of alpha-lactalbumin, FASEB J. 10 (1996) 102–109.
[2] W. Pfeil, Is the molten globule a third thermodynamic state of protein? The example of alpha-lactalbumin, Proteins 30 (1998) 43–48.
[3] E.A. Permyakov, L.J. Berliner, Alpha-lactalbumin: structure and function, FEBS Lett. 473 (2000) 269–274.
[4] K. Nitta, Calcium-binding protein protocols volume 1: reviews and case studies, in: Hans J. Vogel, N.J. Totowa (Eds.), Methods in Molecular Biology, vol. 172, Humana Press, 2002, pp. 211–224.
[5] J.E. Bell, T.A. Beyer, R.L. Hill, The kinetic mechanism of bovine milk galactosyltransferase. The role of alpha-lactalbumin, J. Biol. Chem. 251 (1976) 3003–3013.
[6] M. Svensson, A. Hakansson, A.K. Mossberg, S. Linse, C. Svanborg, Conversion of alpha-lactalbumin to a protein inducing apoptosis, Proc. Natl. Acad. Sci. USA 97 (2000) 4221–4226.
[7] C. Svanborg, H. Agerstam, A. Aronson, R. Bjerkvig, C. Duringer, W. Fischer, L. Gustafsson, O. Hallgren, I. Leijonhuvud, S. Linse, A.K. Mossberg, H. Nilsson, J. Pettersson, M. Svensson, HAMLET kills tumor cells by an apoptosis-like mechanism—cellular, molecular, and therapeutic aspects, Adv. Cancer Res. 88 (2003) 1–29.
[8] W. Fischer, L. Gustafsson, A.K. Mossberg, J. Gronli, S. Mork, R. Bjerkvig, C. Svanborg, Human alpha-lactalbumin made lethal to tumor cells (HAMLET) kills human glioblastoma cells in brain xenografts by an apoptosis-like mechanism and prolongs survival, Cancer Res. 64 (2004) 2105–2112.
[9] L. Gustafsson, I. Leijonhufvud, A. Aronsson, A.K. Mossberg, C. Svanborg, Treatment of skin papillomas with topical alpha-lactalbumin–oleic acid, N. Engl. J. Med. 350 (2004) 2663–2672.
[10] K.H. Mok, J. Pettersson, S. Orrenius, C. Svanborg, HAMLET, protein folding, and tumor cell death, Biochem. Biophys. Res. Commun. 354 (2007) 1–7.
[11] P. Polverino de Laureto, E. Frare, R. Gottardo, A. Fontana, Molten globule of bovine alpha-lactalbumin at neutral pH induced by heat, trifluoroethanol, and oleic acid: a comparative analysis by circular dichroism spectroscopy and limited proteolysis, Proteins 49 (2002) 385–397.
[12] J. Fast, A.K. Mossberg, C. Svanborg, S. Linse, Stability of HAMLET—a kinetically trapped alpha-lactalbumin oleic acid complex, Protein Sci. 14 (2005) 329–340.
[13] M. Svensson, C. Duringer, O. Hallgren, A.K. Mossberg, A. Hakansson, S. Linse, C. Svanborg, Hamlet—a complex from human milk that induces apoptosis in tumor cells but spares healthy cells, Adv. Exp. Med. Biol. 503 (2002) 125–132.
[14] M. Svensson, J. Fast, A.K. Mossberg, C. Duringer, L. Gustafsson, O. Hallgren, C.L. Brooks, L. Berliner, S. Linse, C. Svanborg, Alpha-lactalbumin unfolding is not sufficient to cause apoptosis, but is required for the conversion to HAMLET (human alpha-lactalbumin made lethal to tumor cells), Protein Sci. 12 (2003) 2794–2804.
[15] J. Pettersson, A.K. Mossberg, C. Svanborg, Alpha-lactalbumin species variation, HAMLET formation, and tumor cell death, Biochem. Biophys. Res. Commun. 345 (2006) 260–270.
[16] S.C. Gill, P.H. von Hippel, Calculation of protein extinction coefficients from amino acid sequence data, Anal. Biochem. 182 (1989) 319–326.
[17] K. Kuwajima, K. Nitta, M. Yoneyama, S. Sugai, Three-state denaturation of alpha-lactalbumin by guanidine hydrochloride, J. Mol. Biol. 106 (1976) 359–373.
[18] K. Kuwajima, S. Sugai, Equilibrium and kinetics of the thermal unfolding of alpha-lactalbumin. The relation to its folding mechanism, Biophys. Chem. 8 (1978) 247–254.
[19] S. Ramboarina, C. Redfield, Structural characterisation of the human alpha-lactalbumin molten globule at high temperature, J. Mol. Biol. 330 (2003) 1177–1188.
[20] G. Vanderheeren, I. Hanssens, Thermal unfolding of bovine alpha-lactalbumin. Comparison of circular dichroism with hydrophobicity measurements, J. Biol. Chem. 269 (1994) 7090–7094.
[21] W. Pfeil, V.E. Bychkova, O.B. Ptitsyn, Physical nature of the phase transition in globular proteins. Calorimetric study of human alpha-lactalbumin, FEBS Lett. 198 (1986) 287–291.
[22] K. Yutani, K. Ogasahara, K. Kuwajima, Absence of the thermal transition in apo-alpha-lactalbumin in the molten globule state. A study by differential scanning microcalorimetry, J. Mol. Biol. 228 (1992) 347–350.
[23] Y. Kobashigawa, M. Sakurai, K. Nitta, Effect of hydrostatic pressure on unfolding of alpha-lactalbumin: volumetric equivalence of the molten globule and unfolded state, Protein Sci. 8 (1999) 2765–2772.
[24] M. Mizuguchi, K. Masaki, M. Demura, K. Nitta, Local and long-range interactions in the molten globule state: a study of chimeric proteins of bovine and human alpha-lactalbumin, J. Mol. Biol. 298 (2000) 985–995.
[25] R. Wijesinha Bettoni, C.M. Dobson, C. Redfield, Comparison of the structural and dynamical properties of holo and apo bovine alpha-lactalbumin by NMR spectroscopy, J. Mol. Biol. 307 (2001) 885–898.