Solid-body formation from low moisture ovalbumin powder by pressurization at room temperature

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Summary

Spray-dried ovalbumin powders with moisture contents from 20 to 41 %db (dry basis) were converted into light yellow, transparent fragile solid-bodies by pressurization under 10 to 50 MPa at 30°C. The hardness of the solid-body obtained depended on its moisture content and the hardest solid with a breaking strength of 55 kgf per test piece (11.28 φ × 2 mm) was obtained at 30 %db moisture and pressurization greater than 10 MPa. Solids could not be obtained at moisture contents of greater than 41 %db due to the presence of freezable water. SDS-PAGE and solubility analyses indicated that the ovalbumin molecule was unchanged under these processing conditions. From SEM observations it can be postulated that the mechanism for the solid-body formation is as follows: the globular ovalbumin particle deforms to a flat thin body which is transformed into the transparent solid body with moist ovalbumin powder acting as a plasticizer, gelling agent or a binder of ovalbumin molecules.

Key words: ovalbumin, pressurization, solid-body

Introduction

Natural biopolymers, such as proteins and starches, show interesting behaviors at extreme conditions, i.e. molten flow at an elevated temperature as seen in extrusion and protein gel formation under high pressure at room temperature. In this respect, there is already a large amount of prior art on these phenomena1–7. However, the behavior of protein after mild pressurization at room temperature has failed to be noted. The phenomenon that proteins with low moisture contents changed states from a powder to transparent solid-bodies by relatively low pressurization (<50MPa) at room temperature when cylindrical molded samples were prepared for melt rheology measurements as observed in our previous study8–10 led us to investigate the details of such behavior. In our preparatory experiment, the existence of suitable moisture ranges for the solid-body formation was observed. This fact suggested that the solid-body formation would be strictly related to the water content and physicochemical state of water in the sample protein. Therefore, in the present paper, the solid-body formation from protein powders was investigated in relation to moisture content.

This study is aimed at future utilization of proteins as a biodegradable plastic resource. Because of having a synthetic thermoplastic polymer like behavior, as seen in our
previous study\textsuperscript{11-12}, it is very likely that protein can be processed the same way as synthetic thermoplastics. Additionally, when protein was heated to 140°C and cooled down to room temperature in a completely closed cell, the texture of the obtained materials varied from a hard solid-body at 20 \%db of moisture content to a rubbery-body at 89 \%db. This fact suggested the controllability of physical properties of products solely by adjusting the moisture content. Commodity plastics are usually produced by extrusion, pressure molding, injection molding, melt casting or spinning. Pressure molding will be the method of choice when using powdered protein as the raw material. Therefore, the work described seeks some useful information at the molding step that precedes heat fixing.

Commercial grade Ovalbumin (OVA) was used because of its easy obtainability and high purity, even though it is too expensive for practical use as a biodegradable plastic resource. The aim of this paper is to obtain some basic information on protein behavior under designed mild conditions.

\textbf{Materials and method}

\textit{OVA and its moisture adjustment}

OVA powder (Spray-dried powder, 170 \# mesh pass, commercial grade, Nakarai Tesque, Inc.) containing 8 \%db moisture and 92 \%db protein (Kjeldahl N \times 6.25) was used in this study. OVA was dried at 130°C until the weight of dried material came to equilibrium (over 12 h) and the dried material was defined as absolutely dry matter (moisture content equal to 0). Based on the absolutely dry matter, the moisture content of OVA was indicated as percent on a dried basis. Moisture content of OVA was adjusted to the desired level by adding a calculated amount of finely pulverized ice powder at $-20^\circ$C and thoroughly mixed at ca. $-20^\circ$C. The OVA samples adjusted to moisture contents of 20, 30, 41, 54, 70 and 89 ± 0.2 \%db were prepared and stored in a cold room at $-20^\circ$C until used.

\begin{figure}[h!]
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{Pressure treatment procedure for moisturized ovalbumin. Pressure treatment was performed at 30°C for 20 min under 5-50 MPa of applied pressure.}
\end{figure}
Pressurization
Pressurization of OVA was done by using a flow-tester (Koka Flowtester, Shimazu Co. Ltd., Kyoto, Japan) with a stopper plate in place of a capillary tube. Pre-molded cylindrical OVA (4.0 g) prepared by a hand press were placed in a sample reservoir (11.28 mm in diameter) and then pressurized at desired pressures (10, 30 and 50 MPa) at 30°C for 20 min. The pressure-treated OVA were pull out from the reservoir and used for subsequent analysis. Figure 1 shows the procedure for pressure-treated sample preparations.

Solubility
A 0.5 g of lyophilized OVA (pressure-treated and non-treated) pulverized to pass a 42 # mesh was dissolved with 20 ml of phosphate buffer (0.05 M, pH 7.0) at 30°C for 1 h. Solubility of OVA was determined by the micro-Buret method after centrifugation at 30,000 × g for 20 min at 20°C. Protein solubility was given in percent based on amount of protein in 0.5 g of lyophilized OVA.

SDS-PAGE
Each lyophilized OVA powder was dissolved with phosphate buffer (0.05 M, pH 7.0) containing 2 % SDS and then SDS-PAGE was performed using 15 % polyacrylamide slab gel. After development, the slab gel was stained with Coomassie brilliant blue-R250.

Color measurement
The surface of pressure-treated OVA was polished smooth using # 2000 polishing paper. Color difference (ΔE) of the sample surface was measured based on a standard white tile using a color meter (CR-100, Minolta Co., Tokyo, Japan).

Breaking strength measurement
Figure 2 shows the schematic diagram of breaking strength measurement using a blade shear cell design based on the Kramer Shear Cell. The cell was on a universal testing machine (model : Tensilon-tensile tester UTM-III, Toyo Sokki Co., Tokyo, Japan) through a 100 kgf load cell (T3PI–100L, Toyo Sokki Co., Tokyo, Japan). A two mm thick blade was adopted and the moving blade to stationary blade clearance was adjusted to 0.1 mm by inserting a spacer plate (Fig. 2-E). A pressure-treated OVA was sliced to a 2 mm thickness and placed on the cell. The sample piece was sheared at nine parts and the total sum of working edge-
lengths was 46.6 mm. The peak force of shearing was detected at a cross-head speed of 300 mm/min and the peak force was defined as breaking strength. Measurements were repeated 10 times for each sample and the arithmetic average value was given in kgf/46.6 mm.

**Differential scanning calorimetry (DSC)**

A differential scanning calorimeter (DSC-100, Seiko Instruments Inc., Tokyo, Japan) was used to determine freezable water containing OVA sample. Pressure-treated or non-treated OVA samples were encapsulated in a silver pan and cooled down to ca. −100°C in the calorimeter using liquid nitrogen. The sample pan and an empty reference pan were heated up to 40°C at a programmed rate of 1.5 C/min. Since endothermic peak area, due to melting enthalpy (mJ) of freezable water, gives freezable water content on the basis of unit fusion-enthalpy of pure water (333 J/g), freezable water content in the OVA sample was obtained from the arithmetic average of duplicate or triplicate DSC measurements.

**Scanning electron microscopy (SEM)**

A lyophilized OVA sample was crushed to small pieces (3–4 mm) and a piece was coated with gold in 300 Å thickness. The coated cleavage surface was examined with an electron microscope (ABT-32, Topcon Ltd., Tokyo, Japan) at an accelerating voltage of 15 kV.

**Results**

**Pressure-treated OVA**

Figure 3 (A, B) show typical appearances of non- and pressure-treated OVA (at 50 MPa) containing 30 %db moisture. Both Fig. 3-A and -B were photographs of the sliced pieces in 2 mm thickness. As shown in Fig. 3-A and -B, six black lines placed under the samples were transparent for Fig. 3-B but not for Fig. 3-A. These appearances mean that white opaque OVA powder solidified by a hand press (Fig. 3-A) was transformed to a light-yellow transparent solid-body by the pressurization (Fig. 3-B) at 50 MPa and at 30°C. However, from this photograph, it is not certain that the pressure-treated solid-body (Fig. 3-B) is in a real gel state.

![Fig. 3 Comparative view between (A) non-treated and (B) pressure-treated (50 MPa) ovalbumin with 30 %db moisture.](image)

**Table 1 Solubility of pressure-treated and non-treated ovalbumin**

<table>
<thead>
<tr>
<th>Water content (%db)</th>
<th>Solubility (%)</th>
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<th>50 MPa</th>
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<td>89.6</td>
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Solubility
Solubility of pressure-treated OVA in phosphate buffer did not depend on moisture content and applied-pressure within measuring error (Table 1). From this result, pressurization up to 50 MPa did not seem to denature OVA molecular structure since denatured OVA would show decreased solubility.

SDS-PAGE
Figure 4 shows SDS-PAGE of standard protein (lane A), untreated OVA (lane B), pressure-treated OVA (lane C, 8 %db moisture, applied-pressure 50 MPa; lane D, 30 %db, 50 MPa; lane E, 54 %db, 50 MPa) and purified OVA (Pharmacia, Ltd., grade V, lane F). Even though the observed migration pattern from lane B to E did not show a single band, probably due to the crude grade OVA reagent used, the migration patterns were identical. These results indicate that OVA molecules were not denatured by pressurization within the limit of present pressure-treatment conditions.

Color difference
Figure 5 shows color difference values ($\Delta E$) of pressure-treated OVA with applied-pressure as variable with parameters of moisture content ranging from 8 to 41 %db. As Fig. 5 shows, moisture content markedly affected the $\Delta E$ values. An opaque solid gives lower $\Delta E$ values such as with moisture content of 8 %db while 30 and 41 %db (transparent solid-body) gave considerably high $\Delta E$ values of ca. 50. $\Delta E$ values at 20 %db moisture increased from 11 at 10
MPa to 45 at 30 MPa. Thus 20 %db moisture may give a transition state for moisture-tight solidification of OVA at given applied pressures (10 - 30 MPa). High values of $\Delta E$ may be given a minute-tight transparent structured solid and low values of $\Delta E$ are caused by irregular reflection at the surface of porous solids.

**Breaking strength**

Figure 6 shows the breaking strength of pressure-treated OVA with parameters of applied pressure. The breaking strength of OVA at all treatment pressures gave peak values at a moisture content of 30 %db. At a lower moisture content of 20 %db, the breaking strength of the OVA solid-body depended on treated-pressure and this moisture content may correspond to a transition state from a porous to a minute-tight solid-body. At a moisture content of OVA more than 40 %db, breaking strengths of pressure-treated OVA decreased with moisture content and this phenomenon may be caused by excess moisture. Therefore the minute-tight solid-body of OVA may be obtained with a suitable moisture content of 30 %db.

**Freezable water**

Figure 7 shows freezable water content of pressure-treated OVA as measured by DSC. From the data intercept on the axis of the abscissa in Fig. 7, the maximum unfreezable water content in OVA was determined as 40 %db and moisture greater than 41 %db as freezable water. The amount of freezable water was in proportion to the moisture content of OVA and was independent from applied-pressure on pressurization. Because a hard solid-body of OVA was not formed at more than 41 %db of moisture content, at any applied-pressure, the freezable water may contribute only to form the loose-structure of pressurized OVA.
Fig. 8 Scanning electron photomicrograph of 8 %db moisturized ovalbumin pressure-treated at 30°C. Applied pressure was (A) 0, (B) 10 MPa, (C) 30 MPa and (D) 50 MPa, respectively.

Fig. 9 Scanning electron photomicrograph of pressure-treated ovalbumin with various moisture contents formed by 50 MPa of pressurization at 30°C. Moisture content was (A) 8%db, (B) 20%db, (C) 30%db and (D) 41%db, respectively.
SEM observation

Figure 8 - 9 are SEM micrographs (magnification, 500 × ) showing the influence of pressurization on spray-dried OVA particles. Figure 8 shows pressure effects on OVA powder at a constant moisture content of 8 %db and Fig. 9 indicates the effect of moisture content at a constant applied pressure of 50 MPa. OVA particles disintegrated to more small parts with an increase in applied pressure (Fig. 8-B to D). On the other hand, increase of smooth and minute-tight structured particles of OVA was seen with increase in moisture content (Fig. 9-B to -D). Highly moisturized OVA seemed to be dissolved in water and their phase changed from solid particles to a highly viscous material.

Discussion

The results on color difference measurement (Fig. 5) and break strength measurement (Fig. 6) indicated their dependence on moisture content and the existence of adequate moisture to produce a solid-body by pressure treatment at room temperature. DSC results (Fig. 7) showed the existence of freezable free water above 40 %db moisture. Measured breaking force shown in Fig. 6 indicated a distinct bell shaped curve related to the water content with the maximum value at 30 %db moisture. The descending portion in Fig. 6 was equal to the moisture range at which OVA contains free water, therefore the decrease of shear force at higher moisture range was caused by free water which can be considered to act as the lubricant among the particles and/or molecules. On the other hand, 20 %db moisturized OVA was far from saturation with bound water that is particles might be partly covered by water. Thus the dissolution presumably occurred over limited portions of the particle surface and then OVA became a particle with a solid core surrounded by highly viscous materials which play a role as viscous binders. At 30 %db moisture, the most suitable hydration and dissolution might have occurred by pressurization, leading to the strongest viscous binding as observed in the highest value of shear force obtained. These results indicated that the adequate moisture range for the formation of a transparent solid-body, was from 20 to 40 %db (lower than the maximum bound water content). Although the appearance of samples changed remarkably by pressurization, their solubility showed no decrease (Table 1) and the migration pattern of SDS-PAGE revealed no change in the molecular weight (Fig. 4). Therefore, it is suggested that protein denaturation and structural changes of OVA molecule did not occur under these conditions up to 100 MPa at 30°C. Consequently, solid-bodies obtained could not be classified into the "hydro-gel" category that is usually defined as non-soluble in water with a continuous three-dimensional network, even though they have the appearance resembling a heat induced hydrogel. On the other hand, the microscopic appearance of the solid-body is changed by both moisture content and applied pressure as seen in the SEM observations. These results suggest that the transparent solid-body formation occurred due to the behavior of the sample powder which was not accompanied by any change of molecular structure.

From the above results, the formation mechanism of transparent solid-body is hypothesized as follows: the dissolution of OVA to the surrounding water occurred by pressure
application and a highly viscous material was produced. Subsequently, coagulam are formed by adhesion but the physical state of pressure–treated OVA changed depending on its moisture content from a compressed powder at 8 %db moisture to a highly viscous sol at 54 %db moisture. At this time, unfreezable water (hydrate water) may act on OVA powder as a plasticizer, gelling agent or binder and the formed solid was softened by freezeable water (unhydrated water).

References

室温条件下での加圧による低含水率タンパク質粉末からの Solid-body 形成挙動

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摘 要
低水分含量（8 〜 54%，dry base）に調湿した粗製 ovalbumin 粉体に対し、30℃で50MPaまで加圧処理を行い、固形物の形成挙動を調べた。その結果、20〜41％の試料は黄色味を帯びた光透過性の固形物に変化した。得られた固形物の破壊強度は、含水率によって大きく変化し、不凍水量が最大となる含水率30％dbで最大強度を示した。しかし、自由水が存在する水分含量では、強度が急激に低下した。加圧試料は電気泳動的に未処理物と変化無く、また、溶解度の変化も見られなかったことから、加圧による変性は生じていないと考えられた。SEMによる加圧試料の観察を行ったところ、低含水率の試料では試料粒子を押し固めた状態であったが、破壊強度が最大となり試料では試料粒子間の境界が不明瞭になった。これらの結果から、固形物は、調湿された ovalbumin 粉体の溶解と高粘性物質の形成が加圧によって生じ、形成されたものと考えられた。また、高含水率領域では自由水が滑剤的な働きをして強度を低下させたものと考えられた。