

# Effects of different degradation methods for solubility and bioactive properties of ovomucin

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## Summary

Effects of six different degradation methods for solubility and bioactive properties of ovomucin were studied. The highest solubility of ovomucin was achieved by using sonication (12 min) of the single methods tested, while sonication (6 min) + enzymatic hydrolysis was the best of combined methods. Only three ovomucin samples, namely colloid milling, enzymatic hydrolysis and sonication (6 min) + enzymatic hydrolysis, was found to contain moderate antiviral activity against NDV.

Keywords: ovomucin; enzymatic hydrolysis; sonication; colloid milling; antiviral activity

## Introduction

Ovomucin is a glycoprotein representing about 2-4% of the total egg albumen protein. It is characterized by high molecular weight and a subunit structure. As a possible ingredient to functional foods ovomucin is a particularly interesting protein, because it has been reported to have several bioactive properties, such as immunopotentiator, antitumour and antiviral activity (Ohami et al., 1993; Tsuge et al., 1996a, 1996b; Tanizaki et al., 1997; Watanabe et al., 1998). Ovomucin is easily fractionated from egg white by using isoelectric precipitation. The ovomucin preparation thus obtained is, however, highly insoluble in water or in conventional buffer solutions, which limits its use as a food ingredient. Enzymatic hydrolysis is the method most often used to enhance ovomucin solubility into conventional buffers (Guerin-Dubiard and Brule, 1994 ; Moreau, 1995 ; Moreau et al., 1997; Hiidenhovi et al., 2005; Hammershoj et al., 2008). There are also a few studies in which sonication has been used to solubilizing ovomucin (Hayakawa and Sato, 1976, 1977, 1978; Itoh et al., 1987).

The aim of this study was to explore the effects of different degradation methods for solubility and bioactive properties of ovomucin. Degradation methods studied were enzymatic hydrolysis, sonication, colloid milling, sonication *plus* enzymatic hydrolysis, and colloid milling *plus* enzymatic hydrolysis. Antiviral activity of different ovomucin fractions was determined by using the hemagglutination inhibition test.

## Materials and methods

### Separation of ovomucin

Ovomucin was prepared by using a combination of methods developed by Kato et al. (1970) and by Donovan et al. (1972) with a few modifications. Liquid egg albumen was diluted with three volumes of de-ionized water, stirred for 30 minutes and then adjusted to pH 6 with 2 N HCl. After overnight settling supernatant was siphoned off and the remaining suspension was collected and centrifuged ( $10,000 \times g$ , 20 min, RT) to precipitate crude ovomucin. The crude ovomucin precipitate was washed twice with water by centrifugation and then freeze-dried. Liquid egg albumen used in this study was obtained as a gift from Scanegg Suomi Oy (Piispanristi, Finland).

## Degradation methods

Solubility of ovomucin was studied using six different methods, namely conventional stirring (as a baseline), sonication, colloid milling, enzymatic hydrolysis, sonication *plus* enzymatic hydrolysis, and colloid milling *plus* enzymatic hydrolysis. In all tests phosphate buffered saline (PBS, pH 7.4) and ovomucin concentration 0.2% (2mg/mL) was used. After each dissolving test the ovomucin sample was centrifuged (20,000 x g, 20 min) and aliquots of supernatant were taken, and kept in -20 °C until analysed (except degree of hydrolysis, which was determined at once). All tests were carried out in triplicate.

*Conventional stirring.* Ovomucin suspended in PBS was stirred (3 hr, RT) by using a magnetic stirrer.

*Sonication.* Ovomucin was dispersed in PBS and sonicated for 6 x 1 min or 12 x 1 min at 80W (low) using LABSONIC 2000 Ultrasonic-Homogenizer (B.Braun, Melsungen, Germany). During sonication samples were kept in ice-bath.

*Colloid milling.* Ovomucin suspension was treated (16,000 RPM) for 1 min by using IKA magicLAB equipped colloid mill MK module (IKA WERKE Gmbh & Co., Staufen, Germany).

*Enzymatic hydrolysis.* Ovomucin dispersion was hydrolysed by trypsin (enzyme-substrate ratio (w/w) 1/100) at 45 °C for 3 hr. The enzymatic reaction was terminated by heating (100 °C/6 min).

*Sonication + enzymatic hydrolysis.* After 6 min sonication the ovomucin sample was hydrolysed by trypsin as presented above.

*Colloid milling + enzymatic hydrolysis.* Ovomucin suspended in PBS was treated both by colloid milling and by enzymatic hydrolysis in series. Both treatments were done as described above.

## Characterization of ovomucin samples

Relative solubility (%) was calculated by using the formula

Relative solubility (%) =

$$\frac{\text{protein content (mg) of ovomucin in degraded sample}}{\text{protein content (\%) of crude ovomucin} \times \text{weight (mg) of ovomucin in test sample}} \times 100 \quad (1)$$

Protein content of degraded ovomucin sample was measured by using Bio-Rad protein DC assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA), while protein content of crude ovomucin was determined by using Kjeldahl method (the protein content was calculated by multiplying Kjeldahl nitrogen by factor 6.25).

*N*-acetylneuraminic acid (NANA) content of ovomucin sample was measured by using a periodate-resorcinol method of Jourdian et al. (1971).

Degree of hydrolysis (DH) was determined spectrophotometrically by measuring the number of  $\alpha$ -amino groups released during hydrolysis following reaction with *o*-phthaldialdehyde by the method of Church et al. (1983) as modified by Frister et al. (1988).

Subunit profile and amounts of co-precipitated egg white proteins in ovomucin samples were determined by using Superose 6 gel filtration chromatography (GFC) method described by Hiidenhovi et al. (2002).

Hemagglutination inhibition (HI) activity for Newcastle disease virus (NDV) was determined by using conventional method (Anonymous, 1992). HI-activity was expressed as minimum inhibition concentration (MIC) of a sample protein that completely inhibited virus hemagglutination.

## Results and discussion

As shown in table 1, two treatments, namely sonication (12 min) and sonication (6 min) + enzymatic hydrolysis, resulted in the highest relative solubility (RS) of crude ovomucin. The lowest RS was achieved after conventional stirring (3 hr) as expected, but obtained RS seemed to be abruptly high (57.0%). The reason for this is that the crude ovomucin produced by using isoelectric precipitation (IEP) is a mixture of ovomucin and other co-precipitated egg white (EW) proteins. As can be seen in figure 1A, it was mainly the co-precipitated EW proteins that dissolved into PBS, not ovomucin, during stirring. Also the NANA content of conventional stirring fraction was much lower compared to other ovomucin fractions (table 1), indicating a low amount of ovomucin.

Table 1. Effects of different treatments for ovomucin samples

Treatment	Relative solubility (%)	NANA ( $\mu\text{g}/\text{mL}$ )	Degree of hydrolysis (%)
A) Stirring (3 hr)	57.0	6.2	n.d.
B) Sonication (6 min)	70.6	20.8	n.d.
C) Sonication (12 min)	92.4	30.3	n.d.
D) Colloid milling (1 min)	57.6	18.1	n.d.
E) Enzymatic hydrolysis (3 hr)	73.0	22.7	2.9
F) Sonication (6 min) + enzymatic hydrolysis	92.3	32.2	4.1
G) Colloid milling + enzymatic hydrolysis	71.5	25.9	2.2.

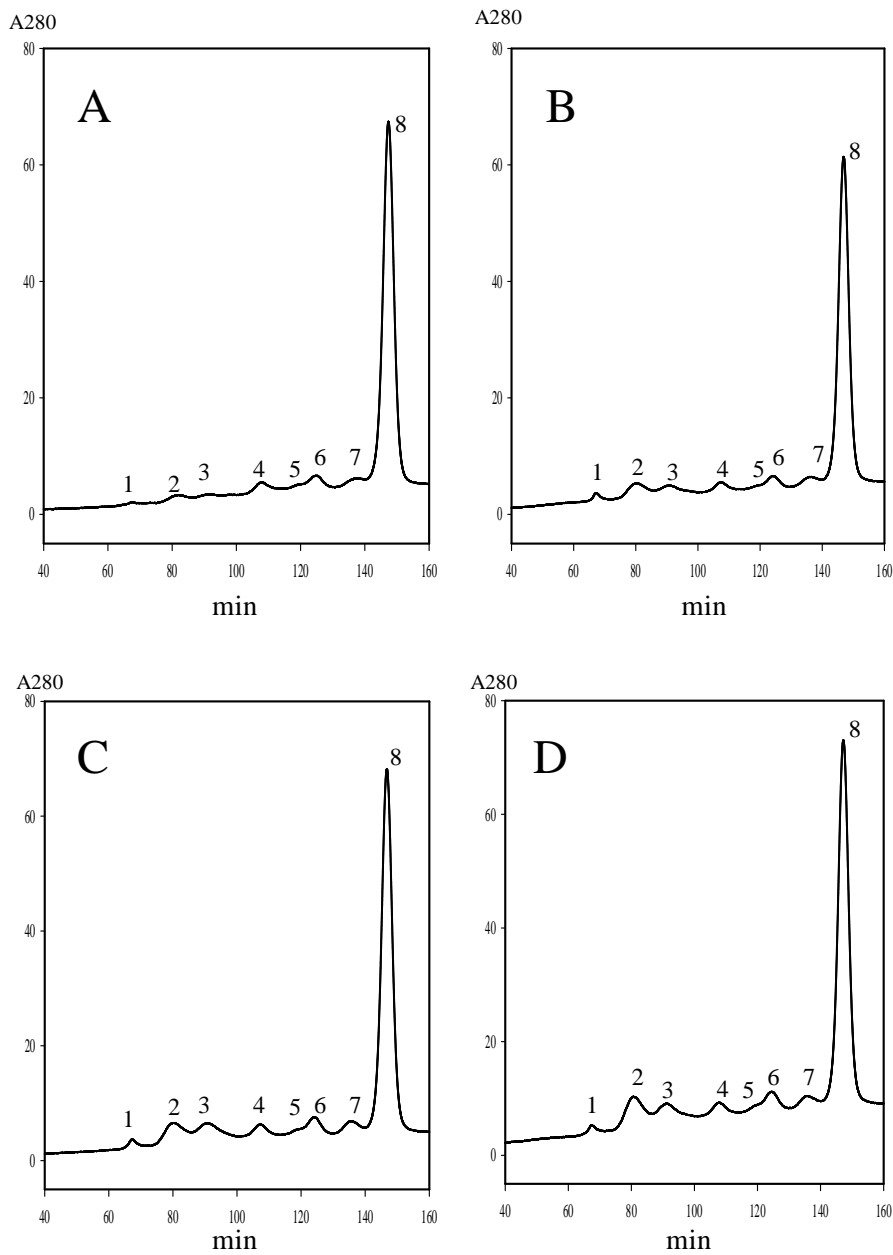
Given values are mean values of triplicate analyses. n.d.= not determined

Thus, it is quite obvious that in the case of the crude ovomucin, the use of any solubility measuring method based on only protein content determination will produce too high readings in some cases.

In this study the DH values of solubilized ovomucin after trypsin hydrolysis remained extremely low (table 1) due to a low E/S-ratio (1/100). In comparison, a DH value 11% was obtained already after 2 hr trypsin hydrolysis when E/S=1/25 was used (Hiidenhovi et al., 2005). Nevertheless, enzymatic hydrolysis seemed to enhance ovomucin

solubilization in every case tested. For example, after 6-min sonication the NANA content was found to be 20.8  $\mu\text{g/mL}$ , while after sonication + enzymatic hydrolysis it was 32.2  $\mu\text{g/mL}$  (table 1).

Figure 1. Elution profiles of degraded ovomucins obtained by Superose 6 HR gel-filtration chromatography. A = conventional stirring, B = colloid milling, C = sonication (6 min), D = sonication (12 min), 1 =  $\beta$ -ovomucin, 2 =  $\alpha$ 1-ovomucin, 3 =  $\alpha$ 2-ovomucin, 4 = ovotransferrin, 5 = unknown (globulins?), 6 = ovalbumin, 7 = ovomucoid and 8 = lysozyme.



Sonication seemed to be a good alternative method for ovomucin solubilisation (figure 1). It was faster compared to hydrolysis and appeared to degradate ovomucin into larger fragments (figure 1C-D).

In colloid milling high-speed shearing forces are used e.g. to disperse and homogenisate mixtures. In this study, a milk-like, smooth and opaque, appearance was attained for crude ovomucin suspension after 1 min treatment by using IKA colloid mill. This "high-speed mixing" favoured also ovomucin solubization into PBS compared to conventional mixing (table 1 and figure 1A-B). The NANA content of ovomucin sample was 6.2 µg/mL after conventional stirring, whereas after colloid milling it was found to be about three times higher (18.1 µg/mL).

Table 2. HI-activity of different ovomucin samples

Treatment	MIC (µg/mL)
A) Conventional stirring	n.i.
B) Sonication 6 min	n.i.
C) Sonication 12 min	n.i.
D) Colloid milling	120
E) Enzymatic hydrolysis	155
F) Sonication + enzymatic hydrolysis	195
G) Colloid milling + enzymatic hydrolysis	n.i.

Given values are mean values of triplicate analyses. n.i.=no inhibition

As can be seen in table 2 only three ovomucin samples (D-F) were found to contain antiviral activity against NDV. The MIC values obtained in this study were higher compared to MIC of 46 µg/mL reported by Tsuge et al. (1996b) indicating lower activity. However, the observed MIC values between these two studies can not be compared straightforwardly, because the virus concentrations used by Tsuge et al. (1996b) was not given.

The reason for HI-activity difference among obtained ovomucin samples is not known. According to Tsuge et al. (1997a, 1997b) the NANA residue in the  $\beta$ -ovomucin contributed greatly to the binding of ovomucin to NDV. However, the ovomucin sample obtained after 12-min sonication contained 30.3  $\mu\text{g}/\text{mL}$  of NANA, about 1.7 times the amount found in the colloid milling sample, still, the latter fraction was the active one. It should be noted that the method used here for NANA determination measured the total NANA content. Therefore it is possible that although sonicated samples had higher NANA content, most of them might be at free form.

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