Isolation of Lysozyme from Hen Egg Albumen by Alcohol-Insoluble Cross-Linked Pea Pod Solid Ion-Exchange Chromatography

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ABSTRACT: Albumen from hen eggs was diluted 3-fold with 0.05 M NaCl solution at pH 4.0 and was further treated with 30% ethanol for 8 h. The supernatant (77900 U/mg protein) thus obtained was further diluted (2.5-fold) with distilled water and its pH value was adjusted to 8.0 before being subjected to alcohol-insoluble cross-linked pea pod solid (AI-CLPPS) ion-exchange chromatography for lysozyme isolation. Results showed that AI-CLPPS ion-exchange chromatography increased the purification to 68-fold with a 72% lysozyme recovery from the starting albumen.

Key Words: hen egg albumen, isolation of lysozyme, alcohol-insoluble cross-linked pea pod solid (AI-CLPPS), ion-exchange chromatography

Introduction

LYSOZYME (MUCOPEPTIDE N-ACETYLMURAMOYLHYDROLASE, EC 3.2.1. 17) is a unique enzyme that catalyzes the hydrolysis of β-1,4-glycosidic linkage of the peptidoglycan in the bacterial cell wall. Lysozyme is also a basic protein with an isoelectric point between 10.5 to 11.5 and is stable under acidic pH conditions, especially when thermally treated (Chang and others 2000).

The antibacterial action of lysozyme is mainly limited to Gram-positive strains. However, some of the Gram-negative strains such as *Escherichia coli* and *Salmonella typhosa* are also sensitive to lysozyme. Sauter and Petersen (1972) found that high lysozyme contents prolonged egg quality. Eggs with high lysozyme contents exhibited better foam stability, fertility, and hatchability than did those with low lysozyme contents. Bakri and Wolfe (1971) have reported that lysozyme can cause destabilization of casein micelles in a manner similar to the action of rennin. Lysozyme also hastens the digestion of milk protein by pepsin (Kisza and others 1974) and plays a significant role in the natural antibacterial activity of milk (Vakil and others 1969). Furthermore, lysozyme can inactivate certain viruses by forming an insoluble complex with acidic viruses (Hasselberger 1978). Thus, lysozyme is used to preserve processed foods such as surimi products (Akashi and Ohno 1972), cheeses (Akashi 1972), fresh vegetables and fruits (Matsuoka 1971). It is also used as an antiinflammatory drug in the treatment of wounds and infections (Proctor and Cunningham 1988).

Lysozyme has become increasingly important in the processing of foods due to its function as a natural food preservative. Therefore, developing a simple efficient methodology for lysozyme production is needed. Various lysozyme-isolating methods have been investigated. Some of these methods are gel permeation chromatography (Palladino and others 1981); membrane separation (Peri and Feresini 1972); affinity chromatography (Weaver and others 1977); ion-exchange chromatography (Cherkasov and Kravchenko, 1969; Li-Chan and others 1986); and a κ-carrageenan method (Yang and others 1998). The classic method for preparing commercial lysozyme is direct crystallization from hen egg white in the presence of 5% NaCl at pH 9.5. That usually requires 1 wk until the enzyme is efficiently recovered. Consequently, another more rapid and efficient method for the partitioning of lysozyme from egg white is needed.

Organic solvents such as ethanol and acetone are frequently used for fractional precipitation of proteins from aqueous solutions as a result of reduction of dielectric constant and due to the increase in the solvating power of the solution (Herskovits and others 1970). Factors affecting the precipitation of proteins in the ethanol solution include temperature, ionic strength, and pH (Harris 1989). On the other hand, the most adequate ionic strength of the protein solution for precipitating proteins with organic solvents was indicated to be 0.05 to 0.2 (Harris 1989). Tribout and Leonis (1975) have studied the effects of methanol, ethanol, and propanol on the H^+ titration curve of hen egg lysozyme and found that the pKa values of aspartic acids (Asp-52 and 101) in the substrate binding sites in 15% alcohol aqueous solutions were higher than those of aspartic acid in water. Those specific interactions between alcohol and active sites of lysozyme might lead to the enhancement of the enzyme activity in alcohol solution.

Deaminated chitin was used as a substrate for the isolation of lysozyme from albumen and exhibited a 36-fold purification of lysozyme with a 99% lysozyme recovery (Weaver and others 1977). The use of such natural material appears to be effective, economic, and handy in the production of lysozyme. Therefore, our study was conducted to develop an improved ion-exchange column chromatography method for the isolation, purification, and concentration of lysozyme using alcohol-insoluble solid (AIS) as the substrate. In the 1st phase, AIS obtained from pea pod were cross-linked (CL). Thereafter, the crude lysozyme solution prepared by the ethanol precipitation method was purified by AI-CLPPS column chromatography. The recovery and the purification results of lysozyme are discussed.

Materials and Methods

Materials

Fresh hen eggs and pea pods (*Pisum sativum*) were purchased from a local market. Albumen was collected by

screening it through a nylon net (2 mm network) to remove the chalazae.

Lysozyme, epichlorohydrin, dimethylsulfoxide, and *Micrococcus luteus* powder were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Taiwan Monopoly Co. Ltd. donated the ethanol (95% v/v).

Pretreatment of albumen with ethanol

An adequate volume (30 mL) of albumen was first diluted 3-fold with 0.05 M NaCl in a beaker with slight stirring for 5 min to reduce the viscosity. The pH value of the diluted albumen solutions was adjusted to 4.0 with 1 N acetic acid. Next, the diluted albumen was homogeneously mixed with an equal volume of 60% (v/v) of ethanol and was then incubated at room temperature for 8 h so as to favor the precipitation of proteins. Supernatants (crude lysozyme solution) were collected by centrifugation (10000 × g, 5 min, 4 °C). Specific activity of lysozyme in the supernatants was determined to be 77900 U/mg protein by the method described further below. Next, the addition of 1 N NaOH adjusted the pH to 8.0. The supernatant was diluted 2.5-fold with distilled water before being applied to AI-CLPPS column described below. Samples from replicated experiments were each analyzed twice for lysozyme recovery with the method described below from albumen.

Preparation of alcohol-insoluble cross-linked pea pod solid (AI-CLPPS)

AI-CLPPS was prepared according to the method described by Inoue and others (1984). One kg of pea pods were homogenized in 80% ethanol in a blender (model NL-3252; Philip Co., Amsterdam, Netherlands) for 3 min, then thermally treated in a water bath at 60 ± 1 °C for 1 h. Solids collected by No. 1 Whatman filter paper were homogenized (Polytron, model CH 6010; Kinematica, Bern, Switzerland) with 5 volumes of 80% ethanol for 1 min. Next, the solids were carefully rinsed with half volume of 95% ethanol and ether. Finally, the solids were vacuum-dried at room temperature for 12 h. Additional grinding of the dried solids in a grinder (BB50; Retsch Co., Berlin, Germany) and straining through a filter (Retsch Co., Berlin, Germany) was done to prepare the AIS. That is, until 36% of AIS did not pass the 80 mesh net and 60% of AIS did not pass the 100 mesh net. About 200 g of AIS was collected from 1 kg of pea pod.

A mixture of 10 g AIS and 5 mL of 50% dimethylsulfoxide was added to 15 mL epichlorohydrin and 10 mL 5 N NaOH. Later, it was incubated in a water shaking bath (model B-202; Firstek Scientific, Taipei, Taiwan) (120 rpm) at 40 °C for 2 h. The collected solids were heated in a boiling water bath for 20 min after being rinsed well with an adequate volume of distilled water, then rinsed again with distilled water and finally treated with adequate volumes of 80% and 90% of ethanol and acetone. The solids, dried at room temperature and here referred to as AI-CLPPS, were refrigerated at 4 °C until needed. The degree of cross-linking for each D-galacturonic acid molecule was between 0.4 to 0.5 (Inoue and others 1984).

AI-CLPPS column chromatography

AI-CLPPS (0.6 g) swelled overnight with distilled water was packed in a column (9.0 cm in length × 1.0 cm in dia). Then it was rinsed with 3 column volumes of 0.5 M NaCl/0.1 M acetate at a flow rate of 35.2 mL/h. First, the column was equilibrated with 3 to 4 column volumes of distilled water. Thus, it was ready to be used. Second, 60 mL of 2.5-fold diluted crude lysozyme solution (pH 8.0) was applied to a AI-CLPPS column. Third, the column was eluted with an adequate volume of distilled water until the absorbance of the eluate at 280 nm was nearly 0.

The column was then eluted with 0.5 M NaCl/0.1 M acetic acid and the eluate was monitored at 280 nm. The total lysozyme activity in the pooled eluate was determined and compared with that of the starting sample to calculate enzyme recovery with the methods described below.

Determination of lysozyme activity

Lysozyme activity in the starting albumen samples, collected supernatants and eluates (AI-CLPPS chromatography) were determined by the turbidimetric assay described by Li-Chan and others (1986) and Weaver and others (1977). *Micrococcus lysodeikticus* cells (Sigma Chemical Co., St. Louis, Mo., U.S.A.) were suspended in 0.067 M phosphate buffer (pH 6.24) at room temperature and the absorbency at 450 nm (A 450) between 0.6 and 0.7 was measured. Thereafter, 2.98 mL of this suspension, 20 μL of enzyme solution containing between 1000 to 2000U/mL activity, was added at 0 time (the time hydrolysis was initiated); then thoroughly mixed with a vortex mixer. The absorbency at 450 nm as compared with the reference buffer solution was recorded every 30 s for 2 min, using a spectrophotometer (Jasco 7800; Spectroscopic Co. Ltd., Tokyo, Japan). A decrease in the absorbency at 450 nm (ΔA 450) of 0.001/min was taken as 1 unit of enzyme activity (U) and the results were expressed as units/mL calculated as:

Activity (U/min) = (ΔA 450/min) / (0.001/min × 0.02 mL)
Specific activity (U/mg) = (U/mL) / (protein mg/mL)
Purification effectiveness = Specific activity of product / Specific activity of starting material

Lysozyme recovery

To compute recovery (%), total lysozyme activity of the collected centrifuged supernatant and of the recovered eluate (AI-CLPPS chromatography) was divided by the total lysozyme activity of the corresponding starting albumen sample. In other words; lysozyme recovery (%) = (total lysozyme activity of the supernatant or of the pooled eluate) / (total lysozyme activity of the corresponding starting albumen sample) × 100%

Determination of protein content

Protein content was determined by the Bradford method (Bradford 1976) with Bio-Rad protein assay dye reagent at 595 nm. Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) (0.2 - 1.4 mg/mL) was used as a standard curve to calculate the protein content.

Results and Discussion

BECAUSE THE ISOELECTRIC POINT (PI) OF MOST OF THE PROteins other than lysozyme and avidin in albumen is between 4.0 to 6.0 (Osuga and Feeney 1977), the pH of the diluted albumen was adjusted to the above range in an attempt to remove the undesired proteins. Both the total protein (mg) and the specific activity (U/mg protein) of lysozyme in the supernatant of the 3-fold diluted albumen varied with the pH of the solution in the preliminary test (data not shown). It was also observed that the specific activity of lysozyme was about 5320 U/mg protein at pH 4.0 (Table 1). Besides, 30% ethanol treatment exhibited a good purification effectiveness (about 15-fold) and a satisfactory recovery (about 80%) of lysozyme after the 3-fold diluted albumen had been incubated for 8 h (Table 1).

Table 1—Purification of chicken egg lysozyme by 30% ethanol treatment and alcohol-insoluble cross-linked pea pod solid ion-exchange chromatography

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification effectiveness[d]	Recovery[e] (%)
Starting material[a]	706030 ± 21100	133 ± 3	5320 ± 150	1.0	100.0
30% ethanol treatment[b]	561850 ± 14230	7.2 ± 0.2	77900 ± 2042	14.6	79.6
AI-CLPPS chromatography[c]	505930±12510	1.4 ± 0.1	361380 ± 10012	67.9	71.7

Average ± standard deviation of three replicates
[a]4 mL of albumen was diluted 3-fold with 0.05 M NaCl at pH 4.0
[b]Supernatant of the starting material treated with an equal volume of 60% ethanol at pH 4.0 for 8 h
[c]Pooled eluate of pea pod CL-AIS chromatography
[d]Purification effectiveness = Specific activity of product / Specific activity of starting material
[e]Lysozyme recovery (%) = (total lysozyme activity of the supernatant or of the pooled eluate) / (total lysozyme activity of the corresponding starting albumen sample) × 100%

AI-CLPPS ion-exchange chromatography

The pH value of the crude lysozyme solution prepared by the above ethanol precipitation method was adjusted to 8.0 in order to decrease the adsorption of proteins other than lysozyme to the AI-CLPPS by changing the charges of most of the proteins to negative. Figure 1 represents the chromatogram of lysozyme by AI-CLPPS column chromatography. The lysozyme activity in the eluate was as high as 43000 U/mL. From Table 1, it was clear that the specific activity, purification effectiveness and the recovery of lysozyme were, respectively, 361380 U/mg, 68-fold, and 72%, when diluted crude lysozyme solution was applied to the AI-CLPPS chromatography column. Similar results were obtained when using apple AI-CLPPS as a substrate (Wang 1998). Affinity chromatography using deaminated chitin as a substrate exhibited a nearly 99% lysozyme recovery and 16300 U/mg specific activity from the 3-fold diluted albumen samples (Weaver and others 1977). The specific activity (361380 U/mg protein) of lysozyme in our study was found to be much higher than that reported by Weaver and others (1977); however, lysozyme recovery was much lower (about 90% from the 30% ethanol-treated sample).

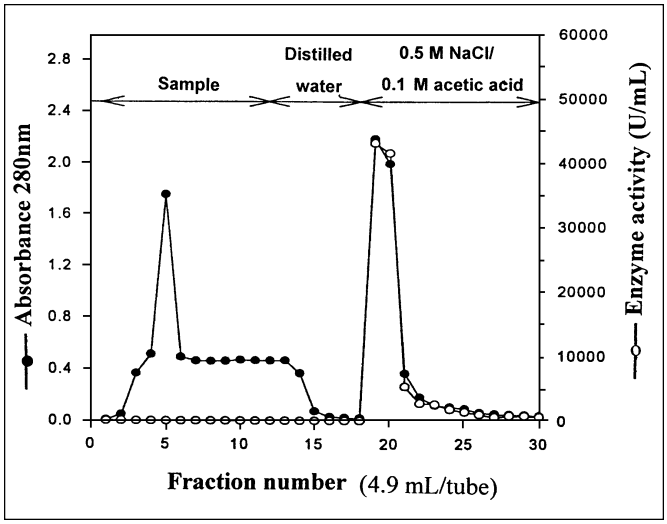

Figure 1—Chromatogram of hen egg lysozyme by alcohol-insoluble cross-linked pea pod solid ion-exchange chromatography. Crude lysozyme solution was prepared by diluting albumen (4 mL) with 3 volumes of 0.05 M NaCl at pH 4.0, followed by 30% ethanol treatment for 8 h and 2.5 dilution with distilled water before being applied to the column. Column: 9.0 ×1.0 cm ; Eluent: distilled water and 0.5 M NaCl/0.1 M acetic acid; Flow rate: 35.2 mL/h; Fractionation: 4.9 mL/tube

The AI-CLPPS column used in our study showed a lysozyme purification of only 39-fold and a 55% lysozyme recovery when albumen was diluted 3-fold with 0.05 M NaCl at pH 4.0 (without ethanol treatment) and the supernatant obtained was applied to the column (data not shown). Treatment of albumen with ethanol was reported to be effective in protecting the lysozyme activity due to the specific reactions between alcohols and active sites of lysozymes (Tribourt and Leonis 1975).

AI-PPS is mainly made up of pectins, which are the polymers of D-galacturonic acid and some monosaccharides such as rhamnose, arabinose, and glucose (Deuel and Stutz 1958). AI-PPS has been used as a substrate to purify polygalacturonase (Jackman and others 1995) and pectinesterase isozyme (Rombouts and others 1979) due to the affinity between pectin and peptic enzymes. In our study, AI-CLPPS was used as the substrate for ion-exchange chromatography by the dissociation of C6 carboxylic acid in the D-galacturonic acid of pectin with a pKa of 3.55 to 4.10 as described by Plaschina and others (1978). The purpose of adding epichlorohydrin to AIS was to strengthen the structure of alcohol-insoluble solid (AIS) by forming the cross-links between pectins (Inoue and others 1984). Moreover, an excessive increase in the links usually decreases the ion exchanging capacity of AIS and thus leads to a decline in the production of the target products. Kohn and others (1976) have stated that the adequate degree of cross-linking for each D-galacturonic acid molecule was 0.4 to 0.5 and 0.20 to 0.25 for pectate used as ion exchange substrates and affinity substrates, respectively.

The dissociation constant of D-galacturonic acid in pectin varies with the sources and the maturity of plant tissues. Additional de-esterification of pectin will increase the number of free carboxylic acid residues in C6 of D-galacturonic acid and therefore enhance cation exchanging capacity. Thus, lysozyme recovery by AI-CLPPS chromatography could possibly be improved by an adequate modification, such as pectin de-esterification.

Conclusions

LYSOZYME WAS RECOVERED FROM ALBUMEN BY AI-CLPPS ion-exchange chromatography, it proved to be an efficient method to isolate lysozyme from hen egg albumen with a satisfactory specific activity (361380 U/mg protein). Pea pod shells were used, which are basically vegetable wastes.

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