Egg White Lysozyme Purification with Sweet Potato [*Ipomoea batatas* (L.) Lam] Leaf Preparations

Wen-Chi Hou and Yaw-Huei Lin*

Institute of Botany, Academia Sinica, Nankang, Taipei 115, Taiwan, Republic of China

Isolation and purification of lysozyme from egg white was improved by utilization of alcohol-insoluble solids (AIS) and cross-linked AIS (CLAIS) of sweet potato [*Ipomoea batatas* (L.) Lam] leaves, an agricultural waste. Egg white was homogeneously diluted 3-fold with distilled water. The lysozyme was isolated and purified by three methods. The first was the batch type of AIS, and products with 28-fold purification and 57% yield were obtained; the second was the batch type of CLAIS column elution with 100 mM acetic acid containing 500 mM NaCl, and products with 74-fold purification and a 59% yield were obtained; the third was CLAIS column elution with linear salt gradients of 0−500 mM NaCl in 10 mM carbonate buffer (pH 9.3), and products with 86-fold purification and 58% yield were obtained.

Keywords: *Agricultural wastes; alcohol-insoluble solids; cross-linked alcohol-insoluble solids; Ipomoea batatas (L.) Lam; lysozyme; sweet potato*

INTRODUCTION

Lysozyme (EC 3.2.1.17; *N*-acetylmuramide glycanohydrase) is an enzyme containing 129 amino acids that splits the (1→4) linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid of cell walls of Gram positive bacteria. It exists broadly in animal tissues and sera, as well as in tears, human and cow's milk, etc. The major commercial source of lysozyme is hen egg white (Proctor and Cunningham, 1988).

Lysozyme purification methods have been reported for many years. The classical method is the crystallization process (Chang et al., 1986a,b) for industrial uses, but it is subject to the drawbacks of time-consumption and low purity and recovery of lysozyme obtained. The second method is a chromatographic process, including ion exchange resins such as Duolite C-464 (Diamond Shamrock Co., Cleveland, OH) (Li-Chan et al., 1986; Durance and Nakai, 1988) and affinity ligands such as chitosan (Weaver and Kroger, 1977; Chang et al., 1986b), tri-*N*-acetylglucosamine (Cornelius et al., 1974; Yamasaki and Eto, 1981), and chitin (Imoto and Yagishita, 1973; Chiang et al., 1993). These resins and ligands need special treatment before being used for lysozyme purification. The more advanced method is ultrafiltration (Chang et al., 1986a; Chiang et al., 1993), but the low purification fold and high cost of the membranes used might limit its applications. In this study, we used the agricultural wastes of sweet potato leaves to prepare alcohol-insoluble solids (AIS) and cross-linked AIS (CLAIS) in order to develop an alternative method for lysozyme purification.

MATERIALS AND METHODS

Materials. Sweet potato leaves and hen eggs were obtained from a local wholesaler. After the yolks had been removed, the egg white was diluted 3-fold with distilled water and homogenized 30 s with a Polytron (Kinematica AG, Switzerland) at low speed in order to avoid foaming. Low molecular weight markers for electrophoresis were obtained from Pharmacia (Uppsala, Sweden). Other chemicals and solvents were from Sigma Chemical Co. (St. Louis, MO).

Preparation of Both Alcohol-Insoluble Solids (AIS) and Cross-Linked AIS (CLAIS) of Sweet Potato Leaves. The preparation of sweet potato leaf CLAIS consists of two steps. The first was AIS preparation, and the second was the AIS cross-linking process. The sweet potato leaf AIS preparation was according to Chang et al. (1993). The AIS cross-linking method was adopted according to Inoue et al. (1984) with some modifications. Cross-linked materials of commercial pectate (CLPA) were used for purification of endopolygalacturonase in the original paper (Inoue et al., 1984). The authors suggested that the final concentration of 15%−35% dimethyl sulfoxide (DMSO) was satisfactory, and 25% DMSO had the maximal yield of CLPA. In this work, much more complex materials of AIS were used instead of only pectate as in the original paper. DMSO of a final concentration of 50% was used in this work to give better swollen effects than 15%−35% DMSO. In addition, the ratios among AIS, epichlorohydrin, and 5 M sodium hydroxide were higher than those of the original paper. A sample of 5 g of sweet potato leaf AIS was placed in 500-mL Erlenmeyer flask. Eighty milliliters of DMSO and 48 mL of epichlorohydrin were added with gentle mixing, and then 32 mL of freshly prepared 5 M sodium hydroxide was added. The mixtures were incubated in a shaker at 160 rpm for 3 h at 40 °C. The mixtures were filtered through Whatman No. 2 filter paper (Maidstone, England), and the residues were washed with 95% ethyl alcohol. After acetone rinsing, the CLAIS were dried overnight at 40 °C.

Purification of Lysozyme Using Sweet Potato Leaf AIS. One gram of sweet potato leaf AIS was weighed and pre-equilibrated with 10 mL of 10 mM carbonate buffer (pH 9.3) in a centrifuge tube overnight. Ten milliliters of diluted egg white was then added to the pre-equilibrated AIS. The mixture was stirred in a centrifuge tube for 30 min and then centrifuged at 10000*g* for 15 min. The supernatant was designated fraction 1. Lysozyme was eluted batchwise for 30 min from pellets with 100 mM acetic acid containing 500 mM NaCl, and then centrifuged at 10000*g* for 15 min; the supernatant containing lysozyme was designated fraction 2.

Purification of Lysozyme Using Sweet Potato Leaf CLAIS. Approximately 400 mg of CLAIS was weighed out, and then 10 mM carbonate buffer (pH 9.3) was added to swell the resins. A column (1.0 × 10.0 cm) was packed with sweet potato leaf CLAIS. After being equilibrated with 10 mM carbonate buffer (pH 9.3), 4 mL of diluted egg white was loaded and washed with 110 mL of the same carbonate buffer. The adsorbed lysozyme was eluted either by batch type with 100

* Corresponding author (fax +886 (2) 782-7954; e-mail boyhlin@ccvax.sinica.edu.tw).

S0021-8561(97)00241-0 CCC: $14.00

Table 1. Purification of Lysozyme from Egg White by Sweet Potato Leaf AIS and Cross-Linked AIS

procedure	total activity unit	total protein (mg)	specific activity (unit/mg)	fold	yield (%)
egg white[a]	66 000	67.0	986	1.0	100
		AIS; Batch Type			
fraction 1	228	60.1			
fraction 2[b]	37 600	1.37	27 400	27.8	57.0
		Cross-Linked AIS Column			
method 1[c]	38 600	0.53	72 830	74.0	58.5
method 2[d]	37 950	0.45	84 330	86.0	57.5

[a] Egg white was mixed with 2 vol of distilled water and homogenized with a homogenizer. [b] Lysozyme was eluted from AIS batchwise with 100 mM acetic acid containing 500 mM NaCl. [c] Lysozyme was eluted from cross-linked AIS column batchwise with 100 mM acetic acid containing 500 mM NaCl. [d] Lysozyme was eluted from cross-linked AIS column with salt gradients of 0−0.5 M NaCl in 10 mM carbonate buffer (pH 9.3).

mM acetic acid containing 500 mM NaCl or with linear salt gradients of 0−500 mM NaCl in 10 mM carbonate buffer (pH 9.3). The flow rate was 55 mL/h, and 5.5 mL was collected for each fraction.

Analytical Methods. Lysozyme activity determination was as according to Shugar (1952) with some modifications by Chang et al. (1986a). One unit of lysozyme activity was defined as lysozyme that lowers by 0.001 absorbance at 450 nm min^{-1}. The protein concentration was analyzed by Bradford (1976) using bovine serum albumin as standards. SDS−PAGE was performed at 15% according to Laemmli (1970), and the gel was stained with Coomassie Brilliant Blue G-250.

RESULTS AND DISCUSSION

Since plant leaves may be an agricultural waste of mass production and hen egg white may be a byproduct of industrial processes, the utilization of AIS from vegetable plants or agricultural wastes for the purification of lysozyme from egg white has double advantages. The aim of this report was to increase the additional values of agricultural wastes.

After fractionation by 80% ethyl alcohol, the soluble sugars and organic acids in filtrates were discarded and the insoluble residues were AIS. The composition of AIS included structural polysaccharides, such as cellulose, hemicellulose, lignin, pectic substances, and structural proteins (Keegstra et al., 1973). The interesting substances in AIS were pectic substances. They were acidic polysaccharides which had ion exchange capacities and existed in cell wall or middle lamella of all plants (Hodge and Osman, 1976). The natural flexibility and strength of the cell wall made AIS suitable ligands for purification aids.

The yield of AIS from fresh sweet potato leaves was about 6.2%. The dissociation constants (pK_a) of pectin ranged from 3.55 to 4.10 (Plaschina et al., 1978), and lysozyme will bind ionically to the carboxyl groups of galacturonate moieties in pectic substances of AIS. Sweet potato leaf AIS were pre-equilibrated with 10 mM carbonate buffer (pH 9.3). After diluted egg white was added to the pre-equilibrated AIS, the mixtures were stirred in a centrifuge tube for 30 min, centrifuged, and the adsorbed lysozyme (fraction 2) was eluted batchwise with 100 mM acetic acid containing 500 mM NaCl. This simple batch type method resulted in a lysozyme preparation with 28-fold purification and 57% yield (Table 1).

After cross-linking treatment with epichlorohydrin in alkaline solution, the CLAIS were more suitable than AIS for column chromatography. The ether linkages between hydroxyl groups of sugar moieties in the cell wall's polysaccharides made CLAIS more resistant to high flow rates after column packing. The 51% yield of CLAIS was obtained from sweet potato leaf AIS. The chromatograms of sweet potato leaf CLAIS for the purification of lysozyme were shown in Figure 1. Figure 1A revealed that lysozyme adsorbed onto the CLAIS column was eluted batchwise with 100 mM acetic acid containing 500 mM NaCl, while Figure 1B revealed that adsorbed lysozyme was eluted with linear salt gradients of 0−500 mM NaCl in 10 mM carbonate buffer (pH 9.3). The use of sweet potato leaf CLAIS for the purification of lysozyme was shown in Table 1. The former method resulted in 74-fold purification and 59% yield, and the latter gave 86-fold purification and 58% yield.

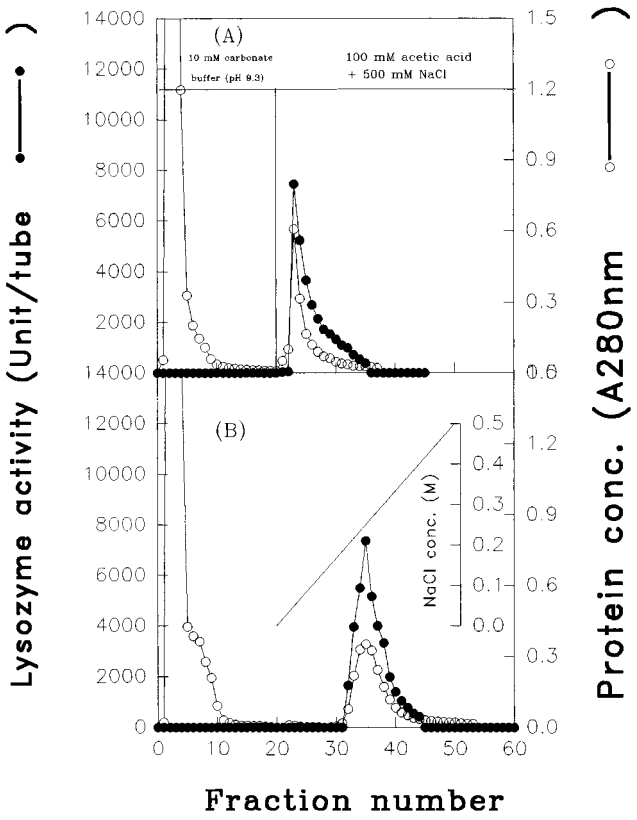

Figure 1. Chromatograms of the sweet potato leaf CLAIS column for the purification of lysozyme from 3-fold diluted egg white. (A) Lysozyme was eluted batchwise with 100 mM acetic acid containing 500 mM NaCl. (B) Lysozyme was eluted with salt gradients of 0−500 mM NaCl in 10 mM carbonate buffer (pH 9.3). Chromatographic conditions: column, CLAIS column (1.0 × 10 cm); elution buffer, 10 mM carbonate buffer (pH 9.3); flow rate, 55 mL/h; fraction size, 5.5 mL.

Figure 2 shows patterns on an SDS−PAGE gel of the lysozyme preparations by AIS and CLAIS. Lane 2 was a commercial lysozyme product (L-6876) from Sigma Chemical Co. (St. Louis, MO), which had been crystallized 3 times. Lanes 3 and 4 were fractions 1 and 2, respectively, which were separated by batch type of AIS. The lysozyme preparation (fraction 2) and commercial one both had some impurities. Lanes 5 and 6 were

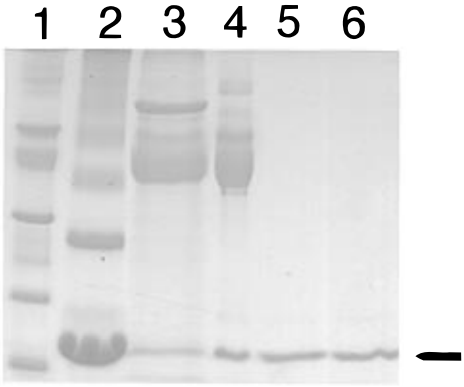

Figure 2. Electrophoretic patterns of lysozyme preparations by sweet potato leaf AIS and CLAIS on a 15% SDS−PAGE gel. Lane 1, low molecular weight markers; lane 2, commercial lysozyme (a product crystallized 3 times, L-6876, Sigma Chemical Co., 30 μg of protein loaded); lane 3 (30 μg of protein loaded) and lane 4 (30 μg of protein loaded) were fraction 1 and fraction 2, respectively, as shown in Table 1, which were separated by batch type of AIS; lane 5 (6 μg of protein loaded) and lane 6 (6 μg of protein loaded), respectively, were lysozyme preparations purified batchwise by CLAIS column with 100 mM acetic acid containing 500 mM NaCl and eluted with salt gradients of 0−500 mM NaCl in 10 mM carbonate buffer (pH 9.3). Arrow indicates the lysozyme position.

lysozyme preparations purified by CLAIS column which were nearly homogeneous.

There is nothing special about sweet potato leaves in particular in the preparation of AIS and CLAIS. Our laboratory has used different parts of sweet potato varieties in different growth stages as test materials since 1980. The availability of sweet potato leaves is the main reason why it was chosen for this research. Theoretically, various plant leaves can be used in the same experiments because pectic substances occur in cell wall or middle lamella of all plants (Hodge and Osman, 1976).

In summary, the utilization of AIS and CLAIS from sweet potato leaves for the purification of lysozyme is a good alternative method. It is easily scaled and valuable for waste utilizations.

ABBREVIATIONS USED

AIS, alcohol-insoluble solids; CLAIS, cross-linking AIS; DMSO, dimethyl sulfoxide; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

ACKNOWLEDGMENT

The authors thank the National Science Council, Republic of China, for financial support (NSC86-2311, B001-0003).

LITERATURE CITED

Bradford, M. M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976,** *72*, 248−254.

Chang, C. T.; Chen, L. H.; Sung, H. Y.; Kao, M. D. Studies on the purification of lysozyme from egg white by ultrafiltration. *J. Chin. Agr. Chem. Soc.* **1986a,** *24*, 86−93.

Chang, C. T.; Jen, J. C.; Sung, H. Y.; Su, J. C. Purification by lysozyme from hen egg white by affinity chromatography. *J. Chin. Agr. Chem. Soc.* **1986b,** *24*, 272−279.

Chang, C. Y.; Tsai, Y. R.; Chang, W. H. Model for the interactions between pectin molecules and other cell-wall constituents in vegetable tissues. *Food Chem.* **1993,** *48*, 145−157.

Chiang, B. H.; Su, C. K.; Tsai, G. J.; Tsao, G. T. Egg white lysozyme purification by ultrafiltration and affinity chromatography. *J. Food Sci.* **1993,** 303−306.

Cornelius, D.; Brown, W. H.; Shrake, A. F.; Rupley, J. A. Purification of native and synthetic lysozyme with tri-(*N*-acetylglucosamine)-agarose. *Methods Enzymol.* **1974**, *34*, 639−645.

Durance, T. D.; Nakai, S. Simultaneous isolation of avidin and lysozyme from egg albumin. *J. Food Sci.* **1988**, *53*, 1096−1102.

Hodge, J. E.; Osman, E. Carbohydrates. In *Principles of Food Science, Part 1, Food Chemistry*; Fennema, O., Ed.; Marcel Dekker Inc.: New York, 1976; pp 41−138.

Imoto, T.; Yagishita, K. Chitin-coated cellulose as an adsorbent of lysozyme-like enzymes. *Agric. Biol. Chem.* **1973**, *37*, 465−470.

Inoue, S.; Nagamatsu, Y.; Hatanaka, C. Preparation of cross-linked pectate and its application to the purification of endopolygalacturonase of *Kluyveromyces fragilis*. *Agric. Biol. Chem.* **1984,** *48*, 633−640.

Keegstra, K.; Talmadge, K. W.; Bauer, W. D.; Albersheim, P. The structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. *Plant Physiol.* **1973,** *51*, 188−196.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970,** *277*, 680−685.

Li-Chan, E.; Nakai, S.; Sim, J.; Bragg, D. B.; Lo, K. V. Lysozyme separation from egg white by cation exchange column chromatography. *J. Food Sci.* **1986,** *51*, 1032−1036.

Plaschina, I. G.; Braudo, E. E.; Tolstoguzov, V. B. Circular dichroism studies of pectin solutions. *Carbohydr. Res.* **1978,** *60*, 1−8.

Proctor, V.; Cunningham, F. E. The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. *CRC Crit. Rev. Food Sci. Nutr.* **1988,** *26*, 359−395.

Shugar, D. The measurement of lysozyme activity and the ultraviolet inactivation of lysozyme. *Biochem. Biophys. Acta.* **1952,** *8*, 302−309.

Weaver, G. L.; Kroger, M. Deaminated chitin affinity chromatography: A method for the isolation, purification and concentration of lysozyme. *J. Food Sci.* **1977,** *42*, 1084−1087.

Yamasaki, N.; Eto, T. A novel adsorbent for affinity chromatography of lysozyme. *Agri. Biol. Chem.* **1981,** *45*, 2939−2941.

Received for review March 24, 1997. Revised manuscript received August 11, 1997. Accepted August 22, 1997.[⊗]

JF970241Z

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1997.