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Novel Cross-Linked Alcohol-Insoluble Solid (CL-AIS) Affinity Gel from Pea Pod for Pectinesterase Purification

MING-CHANG WU,† GUAN-HUI LIN,† YUH-TAI WANG,‡ CHII-MING JIANG,§ AND HUNG-MIN CHANG*,¶

Department of Food Science, National Pingtung University of Science and Technology, Pingtung 912-07, Taiwan, Life Science Center, Hsing-Wu College, 11-2 Fen-Liao Road, Lin-Kou, Taipei 112-44, Taiwan, Department of Sea Food Science, National Kaohsiung Marine University, Kaohsiung 811, Taiwan, and Graduate Institute of Food Science and Technology, National Taiwan University, Taipei 106-17, Taiwan

Alcohol-insoluble solids (AIS) from pea pod were cross-linked (CL-AIS) and used as an affinity gel matrix to isolate pectin esterases (PEs) from tendril shoots of chayote (TSC) and jelly fig achenes (JFA), and the results were compared with those isolated by ion-exchange chromatography with a commercial resin. CL-AIS gel matrix in a column displayed poor absorption and purification fold of PE; however, highly methoxylated CL-AIS (HM-CL-AIS), by exposing CL-AIS to methanolic sulfuric acid to increase the degree of esterification (DE) to 92%, facilitated the enzyme purification. The purified TSC PE and JFA PE by the HM-CL-AIS column were proofed as a single band on an SDS−PAGE gel, showing that the HM-CL-AIS column was a good matrix for purification of PE, either with alkaline isoelectric point (pI) (TSC PE) or with acidic pI (JFA PE).

KEYWORDS: Alcohol-insoluble solids; pectin esterase; pea pod; affinity gel chromatography

INTRODUCTION

Pectin esterase (PE) (EC 3.1.1.11), constantly used in the wine, juice, and other food industries (*1*, *2*), has been found in plants, pathogenic fungi, and bacteria as a methyl ester group hydrolytic enzyme. It removes the methyl ester groups present on the carboxyl moiety of galacturonic acid and produces acidic pectin and methanol for pectin degradation by polygalacturonase and pectate lyase in plant cell walls (*3*−*5*).

Usually, PEs are isolated from plant tissues through a series of procedures such as extraction, ammonium sulfate fractionation, and ion-exchange chromatography or affinity chromatography on a PE inhibitor (PEI)−Sepharose column (*6*, *7*). However, the purification system and gel matrix are costly, and most importantly, lengthy. Therefore, this procedure needs improvement.

The natural flexibility and strength of alcohol-insoluble solids (AIS) fractionated by 80% (v/v) ethanol containing cellulose, hemicellulose, lignin, pectic substances, and structural proteins (*8*) make the dissociated carboxyl groups of pectic substances in AIS suitable ligands for protein purification. Hou and Lin (*9*) used cross-linked AIS (CL-AIS) from sweet potato leaves to isolate hen egg white lysozyme, and the purification fold of the enzyme was as high as 74 with a recovery of 58%. However, higher recovery (72%) was obtained when lysozyme was isolated by pea pod AIS (*10*). On the other hand, cross-linked pectate (CL-PA) treated with ethanol was found available in isolating PE isozymes (*11*), while suffering from some drawbacks such as low purification efficiency due to the strong cation-exchanging property and the relatively low specificity for pectic enzymes.

In the present study, in an attempt to improve the purification efficiency, on the basis of enzyme specificity to the corresponding substrate, an extended experiment was designed using CL-AISs with different degrees of esterification (DE) for PE purification. In addition, the specific activity, purification fold, and electrophoretic homogeneity of isolated PE, with acidic or alkaline pI from different sources, were compared with the corresponding results obtained from the commercial ion-exchange gel matrix.

MATERIALS AND METHODS

Materials. Tendril shoots of chayote (TSC) [*Sechium edule* (Jacq.) Swartz] and pea pod (*Pisum sativum* L.) were purchased from a local supermarket, while jelly fig (*Ficus awkeotsang* Makino) achenes (JFA) were obtained from a local wholesaler in Pingtung County, Taiwan. All chemicals were of analytical grade from Sigma (St. Louis. MO).

Preparation of AIS. AIS was prepared as previously described (*10*, *12*). Briefly, pea pod was homogenized for 3 min in a homogenizer in 80% (v/v) ethanol, and the homogenate was heated at 60 °C in a water bath for 2 h, cooled to room temperature (about 26 °C), and filtered through a Whatman No. 1 filter paper (Maidstone, England). The thus obtained residues were homogenized again with 80% ethanol, filtered,

* To whom correspondence should be addressed. Phone: +886-2-3366-4123. Fax: +886-2-2362-0849. E-mail: Changhm@ntu.edu.tw.
† National Pingtung University of Science and Technology.
‡ Hsing-Wu College.
§ National Kaohsiung Marine University.
¶ National Taiwan University.

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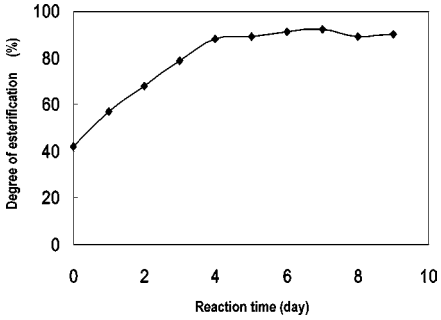

Figure 1. Change in degree of esterification of CL-AIS during methoxylation for up to 9 days. Each value is the average of three determinations.

rinsed with 95% ethanol, and dried under a hood overnight. Additional grinding of the dried solids in a grinder (BB50, Retsch Co., Berlin, Germany) and straining through a filter 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. The thus obtained pea pod AIS was stored in a desiccator until use.

Preparation of CL-AIS. CL-AIS of pea pod was prepared as previously described (*10*) using a method initially described by Hou and Lin (*9*). Ten grams of pea pod AIS was placed in a 500 mL Erlenmeyer flask, to which 150 mL of 40% dimethyl sulfoxide and 40 mL of epichlorohydrin were added with gentle mixing, and then 50 mL of 5 N NaOH was added, followed by incubation in a water shaking bath at 120 rpm for 2 h at 40 °C and filtration through a Whatman No. 1 filter paper. The residues thus obtained were rinsed with distilled water, 80% and 95% ethanol, and acetone in order. After the final acetone rinsing, the solids (CL-AIS) were dried under a hood overnight at room temperature and were stored in a desiccator until use.

Preparation of HM-CL-AIS. Methoxylation of CL-AIS was performed according to the method described by Sajjaanatakul et al. (*13*). Twenty grams of pea pod CL-AIS in an Erlenmeyer flask was added slowly to 40 mL of chilled 2 N methanolic H_2SO_4 in a cold room (about 4 °C) to minimize the evaporation of methanol. Then, the flask was sealed with Parafilm and aluminum foil, and the mixture was then incubated in a cold room for 6 days for methoxylation and subsequently filtered through a Whatman No. 2 filter paper. The thus obtained residues were rinsed repeatedly with methanol to remove the residual sulfuric acid, then rinsed with 80% acetone and acetone in order. After drying under a hood overnight at room temperature, the powder (HM-CL-AIS) obtained was stored in a desiccator until use.

Isolation of TSC PE by CL-AIS, HM-CL-AIS, and CM-Sepharose CL-6B Chromatographies. TSC (50 g) was homogenized first with 4 volumes (w/v) of cold distilled water and then with 4 volumes (w/v) of 0.01 M phosphate buffer (pH 8.0) to remove mostly pectin and water-soluble components. After filtration through a Whatman No. 1 filter paper, the residues were homogenized with 3 volumes (w/v) of 1 M NaCl, filtered through a 6-fold cheesecloth, and centrifuged at 14 000*g* for 30 min. The supernatant thus obtained was fractionated by 40−70% ammonium sulfate, and the precipitates by centrifugation (14 000*g*, 30 min) were dialyzed overnight against deionized water at 4 °C to obtain crude TSC PE.

Subsequently, CL-AIS chromatography (column, 3.0 cm × 10.0 cm; eluent, 0−0.15 M NaCl/0.01 M phosphate buffer, pH 4.0, 5.0, 6.0, and 7.0; flow rate, 20 mL/h; fractionation, 4 mL/tube) was conducted to pool the PE fraction. HM-CL-AIS chromatography was conducted in the same way except the eluent was 0−0.3 M NaCl/0.01 M phosphate buffer (pH 4.0, 5.0, 6.0, and 7.0). For comparison, CM-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) chromatography (column, 2.6 cm × 40.0 cm; eluent, 0−0.5 M NaCl/0.01 M phosphate buffer, pH

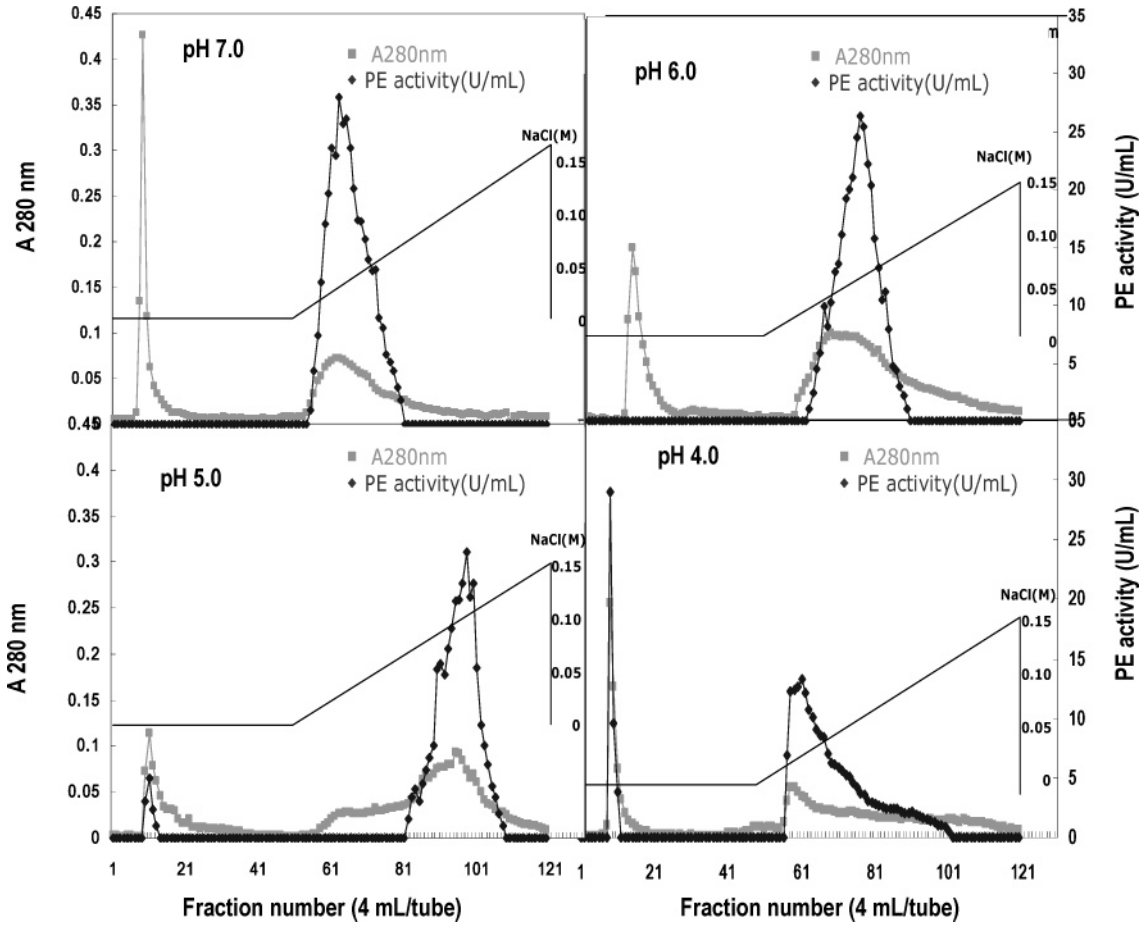

Figure 2. Chromatograms of PE from tendril shoots of chayote on CL-AIS chromatography at pH 4.0, 5.0, 6.0, and 7.0. TSC PE was recovered by 30∼70% ammonium sulfate fractionation. Column, 3 × 10 cm; flow rate, 20 mL/h; elution buffer, 0.01 M phosphate buffer and 0–0.15 M NaCl/0.01 M phosphate buffer.

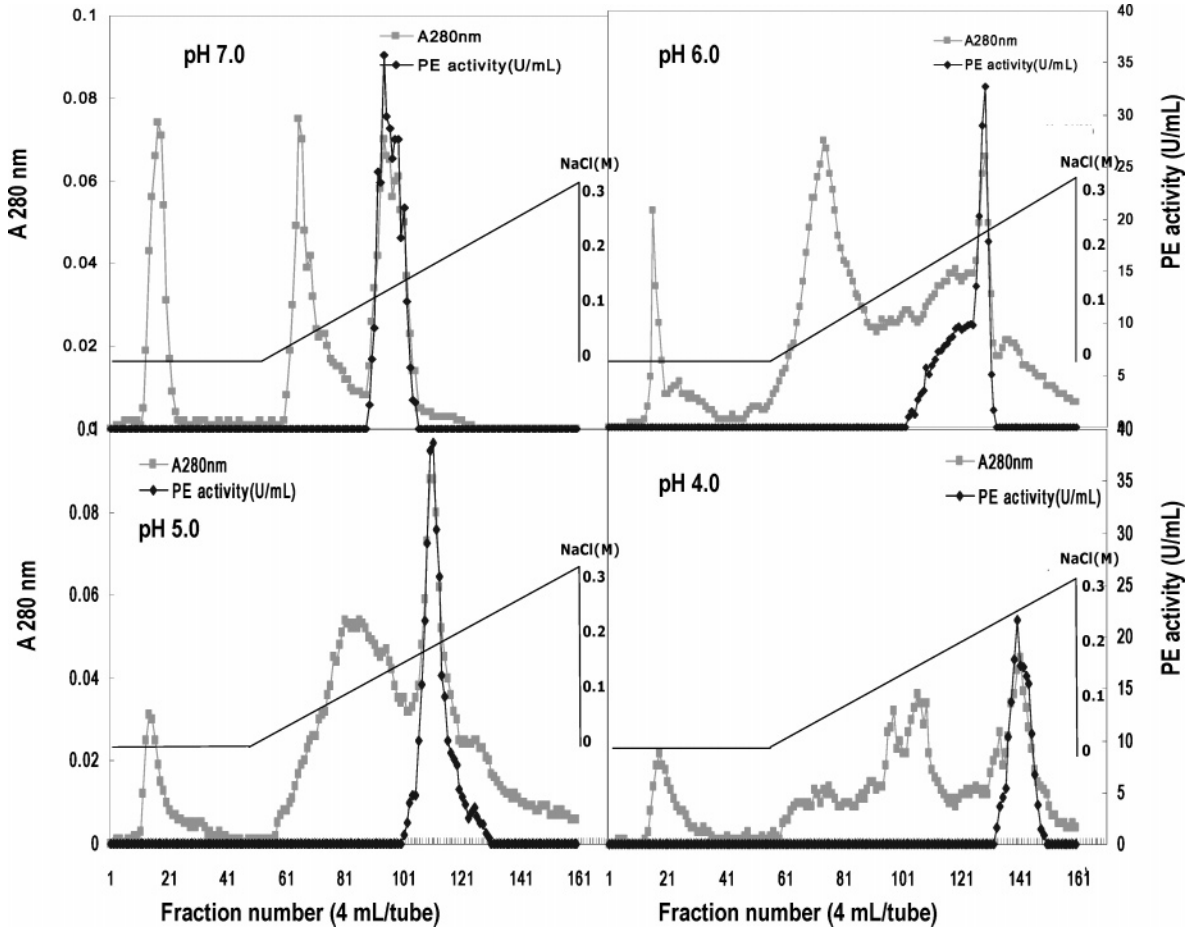

Figure 3. Chromatograms of PE from tendril shoots of chayote on HM-CL-AIS chromatography at pH 4.0, 5.0, 6.0, and 7.0. TSC PE was recovered by 40∼70% ammonium sulfate fractionation. Column, 3 × 10 cm; flow rate, 20 mL/h; elution buffer, 0.01 M phosphate buffer and 0–0.3 M NaCl/0.01 M phosphate buffer.

8.0; flow rate 20 mL/h; fractionation, 4 mL/tube) (*14*, *15*) was conducted to isolate the PE fraction.

Isolation of JFA PE by CL-AIS, HM-CL-AIS, and DEAE-Sepharose Chromatographies. To jelly fig achenes (about 50 g) was added 15 volumes (w/v) of 4% NaCl, and the mixture was stirred gently for 2 h, followed by resting at 4 °C for 22 h to extract PE and filtration through a 6-fold cheesecloth. Proteins in the supernatant were fractionated by 30−70% ammonium sulfate by centrifugation (14 000*g*, 30 min), resuspended, and dialyzed overnight against 0.15 M NaCl/10 mM Tris buffer (pH 7.5) at 4 °C to obtain crude JFA PE solution.

CL-AIS and HM-CL-AIS chromatographies were subsequently conducted as described in the isolation of TSC PE except that the eluent was 0−0.5 M NaCl/0.01 M phosphate buffer (pH 4.0−7.0). For comparison, isolation of JFA PE by DEAE-Sepharose (Pharmacia) chromatography (column, 2.6 cm × 40.0 cm; eluent, 0.15−0.6 M NaCl/0.01 M Tris HCl, pH 7.5) was performed according to the method described by Lin et al. (*16*) and Jiang et al. (*10*).

PE Activity Assay. PE activity in each purification step was measured by continuously titrating the free protons dissociated from the carboxyl groups formed by the PE activity using an automatic pH stat (PH-Stat controller PHM-290, Radiometer, Copenhagen, France). One milliliter of PE solution was added to 15 mL of 0.1 M NaCl/0.5% pectin solution (25 °C) with the pH adjusted to 6.5 immediately before assay. Titration of released protons was carried out with 0.01 M NaOH. One activity unit of PE is defined as the amount of enzyme releasing 1 μequiv of the free carboxyl groups produced by the PE hydrolytic activity on the pectin substrate per min at 25 °C. An enzyme solution previously heated in a boiling water bath for 10 min was treated as a blank. Triplicate samples each were analyzed twice. The following definitions are used: specific activity (*U*/mg) = (*U*/mL)/(protein mg/mL); purification fold = specific activity of enzyme in the sample solution/specific activity of the enzyme in the corresponding crude enzyme solution; enzyme recovery (%) = total enzyme activity in sample solution × 100%/total enzyme activity in the corresponding crude enzyme solution.

Determination of Pectin DE. Degree of esterification of pectin was determined by the method described by Mizote et al. (*17*). Triplicate samples each were analyzed twice.

Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed at 15% gel, and the gel was stained with Coomassie brilliant blue R-250. Low molecular weight maker proteins (97, 66, 45, 30, 20.1, and 14.4 kDa) for gel electrophoresis were the products of Pharmacia.

Protein Determination. Protein content in solution was determined by the Bio-Rad protein assay dye reagent (Bio-Rad, Hercules, CA), using bovine serum albumin (0.2−1.4 mg/mL) as standard ($r^2 = 0.995$).

Statistical Analysis. Analysis of variance of results was carried out using the general linear model procedure of SAS Statistical Software, version 6.11 (*18*). Total activity, total protein, specific activity, purification fold, and recovery of PE were each tested in triplicates. Multiple comparisons of means were carried out by Duncan's multiple range test at $p < 0.05$.

RESULTS AND DISCUSSION

Isolation of TSC PE by CL-AIS, HM-CL-AIS, and CM-Sepharose CL-6B Chromatographies. AIS was treated with dimethyl sulfoxide and epichlorohydrin under strong alkaline condition (5 N NaOH) to perform the cross-linking reaction, and thus, the DE value of AIS was reduced. As shown in **Figure 1**, the DE of CL-AIS was about 40% before methoxylation and

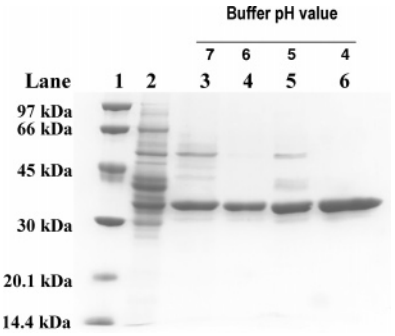

Figure 4. Sodium dodecyl sulfate–polyacrylamide gel electrophoretograms of PE fraction from tendril shoots of chayote. The TSC PE fraction was isolated by HM-CL-AIS column chromatography eluted with 0–0.3 M NaCl/0.01 M phosphate buffer at pH 4.0, 5.0, 6.0, and 7.0. **Lane 1**, marker proteins; **Lane 2**, ammonium sulfate fractionation (40 ~ 70%).

accordingly, CL-AIS acted as a weak cation-exchanger due to the great presence of low methoxyl pectin in AIS.

Ammonium sulfate-fractionated crude TSC PE (with a pI of about 9.0) (*15*) was applied to the CL-AIS column, and the obtained PE chromatogram versus pH value of the eluent is shown in **Figure 2**. The PE fraction was between fraction number 54 and 80 when the column was eluted at pH 7.0; however, it was shifted to a higher fraction number, ranging from 62 and 90, when the column was eluted with the same buffer system at pH 6.0, and to a far higher fraction number (between 81 and 108) when the same column was eluted at pH 5.0. This suggests that the positively charged PE and other proteins are eluted by buffer containing higher ionic strength under lower pH value due to the stronger CL-AIS−PE interactions. This phenomenon is similar to the characteristics of CM-Sepharose cation-exchangers versus pH value.

On the other hand, at pH 4.0, absorption of proteins on the column was poor due to the slight difference between the pK_a value (pK_a 3.55−4.1) of the carboxyl group in pectin and the buffer pH. The mostly nondissociated carboxyl groups at pH 4 were responsible for the low absorption of proteins onto the gel matrix and for the easy elution of PE and other proteins at a very low ionic strength (0.025 M NaCl in 0.01 M phosphate buffer, pH 4.0). The SDS−PAGE results displayed the high impurities in each of the pooled PE fractions in **Figure 2** (data not shown), suggesting that CL-AIS acted mostly as an ion-exchanger. Apparently, the use of the CL-AIS column for PE purification appeared to be limited.

Diluted hen egg white at pH 8.0 with 0.01 M NaCl was subjected to an alcohol-insoluble cross-linked pea pod solid ion-exchange chromatography for lysozyme isolation at pH 8.0 (*10*), and the purification fold was determined to be about 68 with a 72% lysozyme recovery from the starting diluted egg white sample, since lysozyme was the only abundant protein with alkaline pI in egg white. The gel matrix for lysozyme isolation was prepared in the same way as for the TSC PE isolation. Apparently, the ion-exchanging properties of CL-AIS are not suitable for PE isolation in the present study.

HM-CL-AIS Chromatography for TSC PE Isolation. The CL-AIS was methoxylated with methanolic sulfuric acid for up to 6 days to increase the DE value in an attempt to overcome the above-mentioned issues. The DE value of CL-AIS increased from about 40% in the original material to about 88% at day 4 and to about 92% at day 6 during the methoxylation reaction (**Figure 1**). Hence, methoxylation of CL-AIS was conducted for 6 days for the following experiments. Methoxylation at low temperature (4 °C) is to minimize the possible acidic degradation of pectin molecules.

Figure 3 represents the chromatograms of crude TSC PE on the HM-CL-AIS column eluted by buffers at pH 4.0−7.0. PE tends to remove methanol from high methoxyl pectin (HMP), and hence, the increase in the DE of CL-AIS facilitates the AIS−PE interactions. Meanwhile, the increased specificity of PE (enzyme) to pectin (substrate) is considered to be the dominant interaction between HMP in HM-CL-AIS and PE, while the cation-exchanging property of HM-CL-AIS for PE is the minor one due to the decreased free carboxyl groups in HMP. In addition, the pH value of elution buffer solution used is also influential on the charged quantity of HMP matrix for PE isolation.

In comparison to the results in **Figure 2**, each PE fraction in **Figure 3** was pooled at a higher ionic strength when the column was eluted by buffer at the corresponding pH value. For instance, the PE fraction was pooled by eluting the HM-CL-AIS column with 0.25 M NaCl/0.01 M phosphate buffer at pH 4.0 (**Figure 3**), while that was pooled by eluting the CL-AIS column with 0.025 M/0.01 M phosphate buffer at pH 4.0 (**Figure 2**). This could be due to the increased specificity of PE to HMP in CL-AIS and the decreased ionic capacity of CL-AIS. The specificity of PE to HMP is much stronger at pH 4, but it becomes weak as the pH of buffer increases, on the basis of the pK_a value of carboxyl groups in pectic substances. This statement is strongly supported by the SDS−PAGE results in **Figure 4**, in which some other protein bands were detected in the PE fraction eluted by buffer at pH 7.0 (**lane 3**), but they eliminated as the buffer pH decreased to pH 4.0 (**lane 6**). Meanwhile, a PE with a specific activity of about 2260 *U*/mg

Table 1. Purification of PE from Tendril Shoots of Chayote and Jelly Fig Achenes by Three Different Methods after Ammonium Sulfate Precipitation ($n = 3$)[a]

sample	method	total activity (*U*)	total protein (mg)	specific activity (*U*/mg)[b]	purification fold[c]	recovery (%)[d]
tendril shoots of chayote	40 ~ 70% $(NH_4)_2SO_4$	2324 ± 110	12.71 ± 0.51	182.9 ± 7.3	1.00	100.0
	CM-Sepharose CL-6B	1526 ± 70[a]	2.73 ± 0.07[a]	558.8 ± 25.3[b]	3.05 ± 0.13[c]	65.7 ± 3.1[a]
	CL-AIS column (pH 7.0)	1380 ± 60[b]	1.63 ± 0.07[b]	849.4 ± 41.3[a]	4.65 ± 0.20[b]	59.4 ± 2.7[b]
	HM-CL-AIS column (pH 4.0)	672 ± 25[c]	0.30 ± 0.01[c]	2264.0 ± 101[c]	12.38 ± 0.61[a]	28.9 ± 1.2[c]
jelly fig achenes	30 ~ 70% $(NH_4)_2SO_4$	1052 ± 48	5.65 ± 0.24	186.0 ± 8.4	1.00	100.0
	DEAE-Sepharose column	782 ± 34[b]	2.85 ± 0.12[b]	274.0 ± 12.2[b]	1.47 ± 0.07[b]	74.3 ± 3.5[a]
	CL-AIS column (pH 7.0)	807 ± 35[a]	5.11 ± 0.22[a]	157.9 ± 6.7[c]	0.84 ± 0.04[c]	76.7 ± 3.6[a]
	HM-CL-AIS column (pH 7.0)	765 ± 32[c]	0.76 ± 0.02[c]	1002.0 ± 45.2[a]	5.38 ± 0.25[a]	72.7 ± 3.4[a,b]

[a] The pH value in parentheses represents the value at which chromatography is conducted. Triplicate data from separate experiments are expressed as mean ± SEM. Mean values in the same column for the same sample with the different letters are significantly different ($p < 0.05$). Number of samples is *n*. [b] (*U*/mL)/(protein mg/mL). [c] Specific activity of enzyme in sample solution/specific activity of enzyme in crude enzyme solution. [d] Total enzyme activity in sample solution/total enzyme activity in crude enzyme solution.

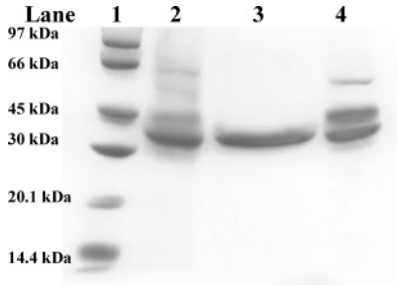

Figure 5. Sodium dodecyl sulfate–polyacrylamide gel electrophoretograms of PE fraction from tendril shoots of chayote isolated by various chromatographies. **Lane 1**, marker proteins; **Lane 2**, CL-AIS chromatography conducted at pH 7.0; **Lane 3**, HM-CL-AIS chromatography conducted at pH 4.0; **Lane 4**, CM-Sepharose CL-6B chromatography conducted at pH 8.0.

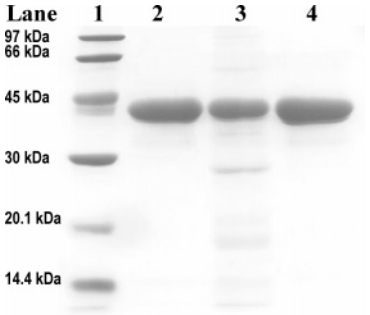

Figure 6. Sodium dodecyl sulfate–polyacrylamide gel electrophoretograms of jelly fig PE fraction isolated by various chromatographies. **Lane 1**, marker proteins; **Lane 2**, HM-CL-AIS chromatography conducted at pH 7.0; **Lane 3**, CL-AIS chromatography conducted at pH 7.0; **Lane 4**, DEAE-Sepharose chromatography conducted at pH 7.5.

protein (**Table 1**) was obtained when it was isolated at pH 4.0. Thus, methoxylation of pectin facilitates the HMP−PE affinity and increases the PE purity during preparation by a HM-CL-AIS chromatography.

In comparison, crude TSC PE was applied to a CM-Sepharose column (chromatogram not shown), and the specific activity and SDS−PAGE of the obtained PE fraction were conducted (**Figure 5**). It was obvious that some proteins other than PE were detected (**lane 4**), and the specific activity of the obtained PE fraction was determined to be about 560 *U*/mg protein (**Table 1**). Apparently, the commercial CM-Sepharose column is inferior to the HM-CL-AIS column in specific activity and purification fold of isolated TSCPE; however, it is higher in PE recovery (about 66%) (**Table 1**). Therefore, on the basis of the above results, the present procedures suggest a cheap, convenient, and, most importantly, effective methodology for high purification of PE by using HMP in CL-AIS.

HM-CL-AIS Chromatography for JFA PE Isolation. JFA PE is a polypeptide with a pI of 3.5 (*16*) or 4.4 (*19*). Both of such acidic PE and CL-AIS (pK_a 3.55−4.1) are negatively charged at pH values higher than the JFA PE pI, and thus, the isolation of JFA PE by the CL-AIS gel matrix at weak pH values appears to be difficult (chromatograms not shown). For instance, the PE fraction pooled by eluting CL-AIS column at pH 7.0 displayed some other protein bands (**lane 3** in **Figure 6**), revealing the impractical use of such column for JFA PE purification.

By increasing the DE of HMP in AIS to 92%, the isolation of acidic JFA PE by HM-CL-AIS column was investigated (**Figure 7**). Similar to the results in **Figure 3** of the HM-CL-AIS column for TSC PE, the JFA PE fraction appeared to slightly shift to a higher fraction number when the column was eluted by a buffer with lower pH value. The required additional ionic strength in the buffer suggested the stronger specificity

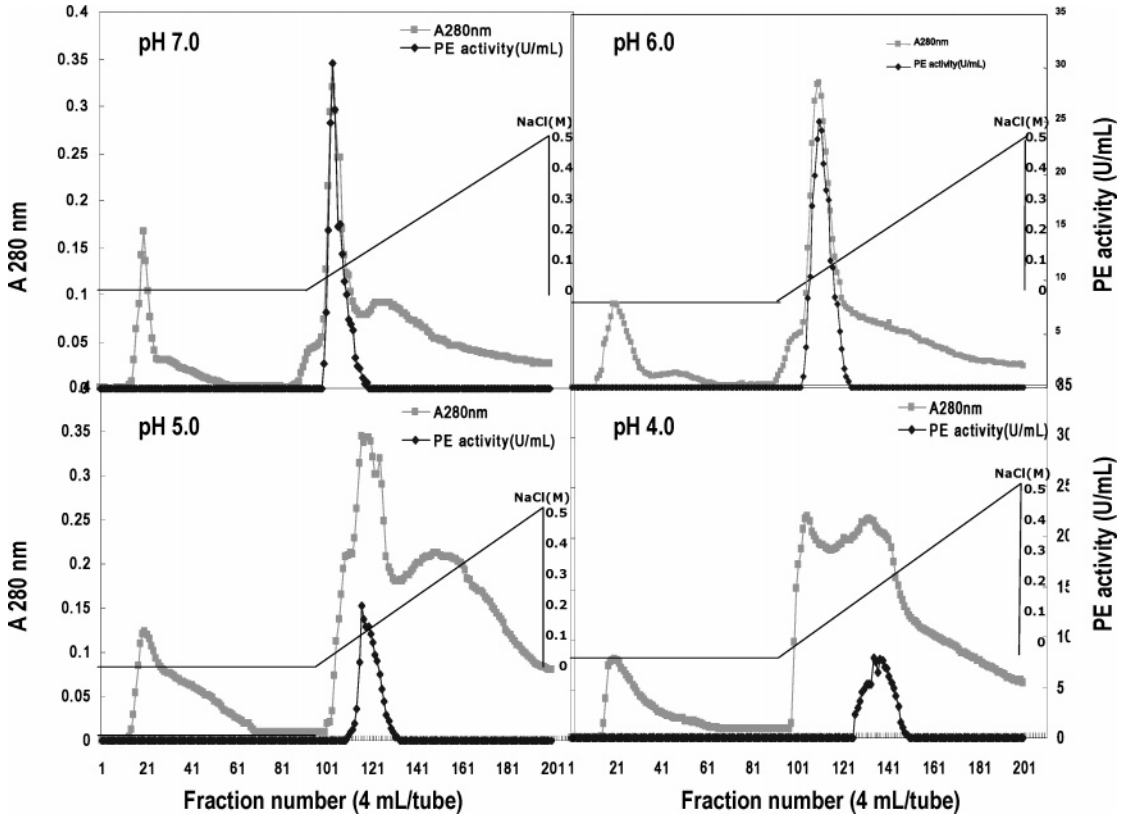

Figure 7. Chromatograms of jelly fig PE on HM-CL-AIS chromatography at pH 4.0–7.0. Jelly fig achene PE was recovered by 30 ∼ 70% ammonium sulfate fractionation. Column, 3 × 10 cm; flow rate, 20 mL/h; elution buffer, 0.01 M phosphate buffer and 0–0.5 M NaCl/0.01 M phosphate buffer.

of PE to HMP at lower pH value. However, the JFAPE isolated by the HM-CL-AIS column at pH 7.0 displayed electrophoretic homogeneity in an SDS−PAGE gel (**lane 2** in **Figure 6**), which was also observed in all PE fractions isolated by column at other pH values (pH 4.0−6.0) (data not shown). This could be due to the dominant specificity of JFA PE to HMP in HM-CL-AIS under the tested experimental conditions (pH 4−7), regardless of the relatively weak ion-exchanging properties of the matrix at neutral pH value. In addition, the recovery (%) and specific activity of JFAPE was about 73% and 1000 *U*/mg protein, respectively, when the HM-CL-AIS column was eluted at 7.0 (**Table 1**). The specific activity of PE was likely to be higher when the chromatography was conducted at lower pH values. Thus, the developed method of the HM-CL-AIS column for the purification of PE, with either high or low pI, appeared to be effective.

Comparison of PE homogeneity with that isolated by commercial DEAE-Sepharose gel was also conducted (**lane 4** in **Figure 6**). No apparent difference between **lane 2** (HM-CL-AIS) and **lane 4** (DEAE-Sepharose) in **Figure 6** was observed. However, the specific activity of the obtained PE was only 274 *U*/mg protein, lower than that obtained by HM-CL-AIS, while the recovery was similar between the two systems. The molecular mass of JFAPE was determined to be 42.5 kDa on the basis of the results in **Figure 6**.

Affinity chromatography of the PEI−Sepharose column was used as a substitutive step of heparin-Sepharose or DEAE-Sephadex for PE purification (*6*, *7*). However, the purification system including CNBr-Sepharose resin, kiwi fruits, and PEI purification are more costly than that of the HM-CL-AIS column. The method developed in the present study proved to be much more convenient and easier for the isolation of PE. Hence, utilization of HM-CL-AIS from agricultural waste such as pea pod is a good alternative method for PME purification.

ABBREVIATIONS USED

TSCPE, tendril shoots of chayote PE; JFAPE, jelly fig achene PE; CL-AIS, cross-linked alcohol-insoluble solid; HM-CL-AIS, highly methoxylated CL-AIS.

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