# Separation and Utilization of Pectin Lyase from Commercial Pectic Enzyme via Highly Methoxylated Cross-Linked Alcohol-Insoluble Solid Chromatography for Wine Methanol Reduction

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The isolation and utilization of pectin lyase (PL) from commercial pectic enzyme for methanol reduction in wine production was investigated. PL can be separated from pectinesterase (PE) and polygalacturonase (PG) on HM-CL-AIS affinity chromatography at pH 4; however, it is difficult to further distinguish PE from PG. Some desirable physicochemical properties such as transmittance, lightness, redness, and lower total pectin content are found in the external enzyme adding groups (PL, PE and PG, and pectic enzyme groups) in comparison to the control group. Methanol contents in pectic enzyme and the PE and PG groups increase from $628 \pm 13$ (control group) to $3103 \pm 16$ and $1736 \pm 67$ mg/L ethanol in the final products, respectively. Nevertheless, the adding of PL does not cause any increase in methanol content. The results present in this study suggest that the HM-CL-AIS column is a simple, inexpensive, convenient, and effective method for PL purification. Moreover, the partial purified PL is a potential replacement of commercial pectic enzyme for pectin depolymerizing, methanol content reducing, and wine quality improving in wine production.

## INTRODUCTION

Methanol, a toxic compound to humans by ingestion or even inhalation, can be produced from the hydrolysis of methyl ester groups in pectin by PE. Although methanol will occur naturally at a low level in fresh fruit juices as well as most alcoholic beverages (such as beer, wine, and distilled spirits) without causing any harm to the human body, a high quantity of methanol is produced in grape wine, carambola wine, and some distilled fruit spirits due to the action of PE on pectin during the mashing, fermentation, and aging stages (*1–4*).

The legal limit for naturally occurring methanol to prevent danger to public health in Taiwan is 2000 mg of methanol/L of ethanol for red wine and 1000 mg of methanol/L of ethanol for other alcoholic beverages, while the general limit from the EU for naturally occurring methanol (10 g of methanol/L of ethanol) provides a greater margin of safety (*5*). However, illicit drinks made from industrial methylated spirits, 5% (v/v) methanol/ 95% (v/v) ethanol, can cause severe and even fatal illness (*5*).

Hence, the safety improvement of wine and spirits by the inhibition of methanol production is recommended in the processing of wine and liquor to avoid methanol contamination before consumption.

In the food industry, the presence of pectin in fruit juices causes an increase in viscosity, thereby impeding the processes of filtration, concentration, and clarification of fruit juice (*6*). Therefore, pectin depolymerizing enzymes are widely used for the treatment of pectin compounds in the fruit and vegetable processing industries (*7*).

The most common commercial pectic enzyme used today is a complex of PE, PG, and PL, etc. from *Aspergillus niger*, *Penicillium dierckxii*, *Kluyveromyces maxianus*, and *Penicillium griseoroseum* (*8, 9*). It can be classified into two main groups based on their attachment on the galacturonan backbone of the pectic molecule, including a de-esterifying enzyme (PE), which removes a methoxyl group from pectin, and depolymerizing enzymes (i.e., PL and PG), which cleave the bonds between galacturonate units. The commercial pectic enzyme plays an important role in the winemaking process and has been used in wine industries to improve fruit juice and wine must for the purposes of extraction, clarification, and filtration, thus increasing the yield and quality, such as pigment, flavor, transmittance,

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and viscosity (*10*−, *11*). However, the use of commercial pectic enzyme suffers from the major drawback of high methanol content in some wine products (*2*−*4*).

Highly methoxylated cross-linked alcohol-insoluble solid (HM-CL-AIS) affinity gels prepared from pea pods were reported to purify PE (*12*). Therefore, it is possible to remove PE and isolate PL from commercial pectic enzymes by HM-CL-AIS in industrial processing for a low methanol production. The effect of HM-CL-AIS affinity column on the separation of PE, PG, and PL from commercial pectic enzymes was performed in the present study. Consequently, the separated enzymes were added to grape must to compare its effects on physicochemical properties during fermentation and the decrease of methanol content in the final product.

## MATERIALS AND METHODS

**Materials.** Fresh Black Queen (*Vitis vinifera* × *V. labrusca*) was purchased from a local supermarket of Taichung County in Taiwan. Commercial liquid pectic enzyme (Peclyve CP) from a microbial source and commercial wine yeast RA-17 (*Saccharomyces cerevisiae*) were purchased from Lallemand Australia Pty. Ltd. (North Adelaide, Australia). Citrus pectin with a degree of esterification (DE) of 90−93% and methanol was purchased from Sigma (St. Louis, MO). Citrus pectin with DE 60−66%, D-galacturonic acid, and polygalacturonic acid was purchased from Fluka (Buchs, Switzerland). The 95% ethanol was purchased from Taiwan Tobacco and Liquor Corporation, Taipei, Taiwan.

**Preparation of HM-CL-AIS.** HM-CL-AIS of pea pods was prepared according to the method described by Wu et al. (*12*). Alcohol-insoluble solids (AIS) of pea pods were prepared followed by homogenization in 80% (v/v) ethanol, heating, cooling, and filtration. The obtained residues were homogenized again with 80% ethanol, filtered, rinsed with 95% ethanol, dried under a hood overnight, and then ground in a grinder (BB50, Retsch Co., Berlin, Germany) until 36% of AIS did not pass the 80 mesh net and 60% of AIS did not pass the 100 mesh net. The obtained AIS were stored in a desiccator until use.

Subsequently, 10 g of AIS was stirred gently into a mixture of 150 mL of 40% dimethyl sulfoxide and 40 mL of epichlorohydrin, and then 50 mL of 5 N NaOH was added, followed by 2 h incubation at 40 °C and filtration. 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.

Finally, HM-CL-AIS was prepared from 20 g of CL-AIS, which was placed in an Erlenmeyer flask, then 2 L of chilled 2 N methanolic $H_2SO_4$ was slowly added in a cold room. The mixture was incubated in a cold room for 6 days for methoxylation followed by filtered through Whatman No. 2 filter paper. Thus obtained residues were rinsed repeatedly with methanol to remove the residual sulfuric acid and 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.

**Separation of PE, PG, and PL using HM-CL-AIS.** HM-CL-AIS chromatography was performed according to the method described by Wu et al. (*12*). A total of 0.2 mL of commercial pectic enzyme was applied on HM-CL-AIS column (2.5 cm × 20.0 cm; flow rate, 40 mL/h) for separation. The column was equilibrated with either 0.01 M acetate buffer (pH 4 and 5) or 0.01 M phosphate buffer (pH 6 and 7) and then eluted with the same buffer at 0−1 M Nacl gradient for PE, PG, and PL separation. The 4 mL/tube fractions were collected and assayed for the activities of PE, PG, and PL.

**Determination of PE Activity.** PE activity was determined according to the method described by Jiang et al. (*13*). A total of 15 mL of 0.1 M NaCl/0.5% citrus pectin (DE 60−66%) solution (25 °C), with the pH adjusted to 6.5 immediately before assay, was mixed with 1 mL of PE solution. The PE activity was determined by titrating (PH-Stat Controller PHM-290, Radiometer, Copenhagen, Denmark) the free protons dissociated from the free carboxyl groups formed by the PE

activity, and the volume of 10 mM NaOH required to maintain a pH of 6.5 of the reaction solution at 25 °C in a water bath was recorded within 5 min. One enzyme activity unit represents 1 μeg 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.

**Determination of PG Activity.** PG activity was determined by measuring the release of reducing groups from citrus pectin using a 3,5-dinitrosalicylic acid (DNS) reagent assay according to Muchuweti et al. (*14*) with some modification. The mixture obtained from the reaction containing 0.8 mL of 0.3% citrus pectin (DE 60−66%) in 0.2 M acetate buffer, pH 4 and the enzyme solution (0.2 mL) was incubated at 37 °C for 30 min and terminated by heating the mixture in a boiling water bath for 5 min. In the control tubes, the substrate 0.3% citrus pectin was added after the heat treatment. The formation of the reducing group was measured by adding 1 mL of 3,5-dinitrosalicylic acid (DNS) (Sigma) solution and then was boiled for 10 min. After cooling, 3 mL of distilled water was added, and the absorbance of the resulting colored mixture was monitored at 540 nm (*15*). One unit of PG activity is defined as the amount of enzyme catalyzed by the liberation of 1 μmol of galacturonic acid per min at 37 °C.

**Determination of PL Activity.** PL activity was assayed spectro-photometically according to Bai et al. (*8*) and Chen et al. (*16*) with some modification. PL activity was determined by measuring the increase in absorbance at 235 nm of a reaction mixture containing 0.2 mL of 0.5% pectin (DE 90−93%) in 10 mM Tris-HCl buffer (pH 8) plus 0.2 mM $CaCl_2$ and 0.2 mL of enzyme at 40 °C. One unit of PL activity is defined as the activity that caused an increase in absorbance of 0.005 per hour at 235 nm under the reaction conditions.

**Protein Determination.** Protein concentrations were determined by the BIO-RAD Protein Assay (Bio-Rad), using bovine serum albumin as a standard.

**Preparation of Wine.** The red grapes (7.5 kg) were cleaned with distilled water and crushed to obtain grape musts, to 1 kg of which sucrose and sodium-pyrosulfite were added to reach 24 °Brix and 100 ppm (as $SO_2$), respectively. Grape must was subsequently divided into five groups containing different kinds of pectic enzymes before yeast inoculation: (i) control group: without external enzyme adding; (ii) pectic enzyme group: with commercial pectic enzyme solution (containing 263.0 units of PE, 340.5 units of PG, and 399.6 units of PL); (iii) PE and PG group: with partial purified PE solution (containing 265.0 units of PE, 183.0 units of PG, and 27.8 units of PL); (iv) 1× PL group: with partial purified PL solution (containing 353.6 units of PL and 13.3 units of PG); and (v) 3× PL group: with 3-fold concentration of partially purified PL solution of the 1× PL group (containing 1060.8 units of PL and 39.9 units of PG).

Commercial wine yeast RA-17 (0.25 g) was activated previously in 25 mL of warm water (40−43 °C) for 15 min to make a suspension and then was added to 1 kg of the grape must. Fermentation was conducted at room temperature (25 ± 2 °C) for 15 days. During fermentation, sampling was conducted every 3 days to determine the changes in physicochemical properties after centrifugation at 13 000*g* for 20 min at 4 °C.

**Determination of Total Soluble Solids.** A hand-held refractometer (N-1E, Atago, Tokyo, Japan) was used to determine the TSS (as °Brix) of semi-products and wines. The refractometer was adjusted with distilled water each time before use.

**Determination of pH Value.** Changes in pH value of semi-products and wines during fermentation were determined by a pH meter (PHM 290, pH stat, Radiometer, Copenhagen, Denmark) at room temperature.

**Specific Gravity.** Specific gravities of semi-products and wines were determined according to the method described by Wu et al. (*3*), to determine the specific gravity of semi-products and wines at 20 °C.

**Determination of Titratable Acidity.** Distilled water (25 mL) was added to the semi-product or wine (5 mL), and then the pH value was brought to 8.1 by adding 0.1 N NaOH. The volume (milliliters) required to bring the pH to 8.1 by a pH stat was recorded. Tartaric acid was used as a standard.

**Color Analysis.** Color changes of semi-products and wines during fermentation were monitored for Hunter's *L* (brightness), *a* (redness−

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greenness), and *b* (yellowness−blueness) by a Spectro-Colorimeter (HunterLab color Quest XE, HunterLab, Reston, Virginia).

**Determination of Transmittance.** Spectrophotometer (U-2001, Hitachi, Ltd., Tokyo, Japan) was used to determine the transmittance of semi-products and wine at 660 nm. Samples were centrifuged (13 000*g*, 20 min) before determination.

**Determination of Pectin.** Pectin of semi-products and wines during fermentation was determined according to Jiang et al. (*17*). Pre-cooled $H_2SO_4$ (2 mL) and distilled water (15 mL) were slowly added into 5 mg of AIS of semi-products and wines. The mixture was filtered with Whatman No. 1 filter paper to obtain the pectin solution after magnetic stirring for 1 h in an ice bath. An adequate volume (0.5 mL) of the pectin solution was mixed with 3 mL of 12.5 mM sodium-tetraborate solution (in sulfuric acid) in an ice bath and then heated in a boiling water bath for 5 min. After cooling, the reaction mixture was mixed well with 0.05 mL of 0.15% *m*-hydroxydiphenyl solution in 0.5% NaOH and then rested for 5 min. The absorbance at 520 nm was recorded. D-Galacturonic acid was used to construct the standard curve for the calculation of pectin content in the samples.

**Determination of DE.** DE of pectin in semi-products and wines was determined according to the method described by Jiang et al. (*18*) with some modifications. A total of 10 mg of AIS from semi-products and wines was added to 10 mL of 0.5 N KOH, followed by the processes of incubation (at ambient temperature for 1 h), filtration, and neutralization (with dilute phosphoric acid to pH 7.5) and then the volume was brought to 25 mL using 0.05 M phosphate buffer (pH 7.5). Subsequently, gas chromatography (GC) was used to analyze the methanol content of the thus obtained solution. The DE of pectin was determined by the following equation:

$$DE (\%) = (\text{methanol content} \times 31/32 \div 16.32 \div \text{pectin content})100\%$$

**Determination of Methanol and Ethanol.** The contents of methanol and ethanol in samples were determined according to the method described by Wang et al. (*19*) with some modifications. Semi-products and wine were centrifuged first and then pressed through a 0.45 $\mu$m membrane filter (Millipore, Concord, MA) for GC analysis. This analysis of methanol and ethanol was conducted in a Trace 2000 GC (TermoQuest, Milan, Italy) equipped with computer integrator software (Chrom-Card version 4.01 for Trace GC, TermoQuest, Milan, Italy), a 30 m CP-Wax 52 CB megapore capillary column (0.53 mm i.d. and film thickness, 1.5 mm; ChromPack, Palo Alto, CA), and a flame ionizing detector (H$_2$: 30 mL/min and air: 300 mL/min). The flow rate of carrier gas nitrogen was set at 5 mL/min. The temperatures at injector port and detector were set at 210 and 280 °C, respectively, and splitless injection (about 0.5 $\mu$L for each injection) was used. The oven temperature was controlled with a temperature elevation program during analysis, which was initially set at 38 °C for 3 min, then elevated to 250 °C at the rate of 50 °C/min and maintained for 1 min. Methanol (or ethanol) and the internal standard acetonitrile solution were mixed in the following combinations: 20:1, 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, 1:20, and 1:50 (v/v) etc. and then subjected to GC analysis for the relative response factor (RRF) determination. Subsequently, the content of methanol (or ethanol) for each sample was determined according to the peak area of methanol (or ethanol) and acetonitrile obtained from GC analysis and the RRF value.

**Statistical Analysis.** Statistical analysis was accomplished using SAS Statistical Software, version 6.11 (SAS Institute). The triplicate samples were analyzed twice in this study. The difference between the means was analyzed using Duncan's multiple range tests.

## RESULTS AND DISCUSSION

**HM-CL-AIS Chromatography for PE, PG, and PL Separation.** Commercial liquid pectic enzyme from the microbial source was applied onto a HM-CL-AIS column, and thus, the obtained enzyme separation chromatogram versus pH value of the eluent was investigated. To separate PE, PG, and PL in a simple step, activity chromatograms of these enzymes were compared to one another. Because of the dominant specificity
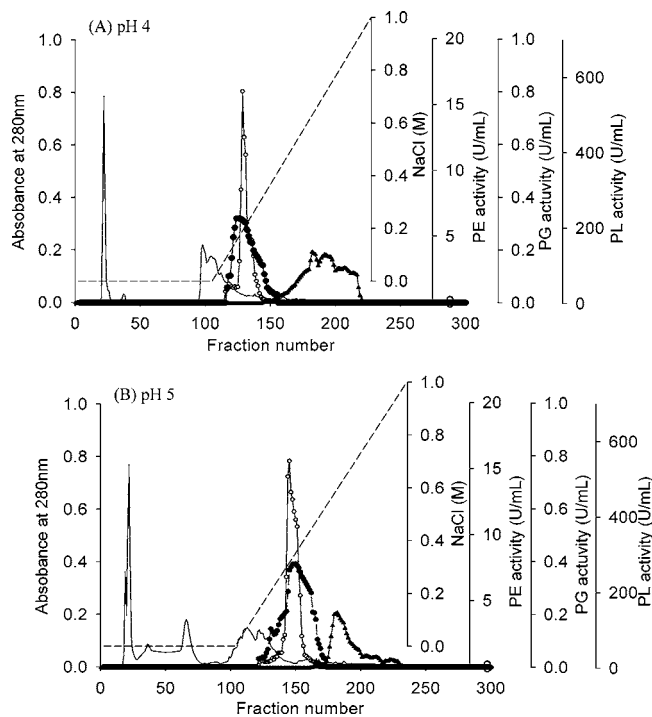


**Figure 1.** Chromatogramphic patterns of PE (○), PG (●), and PL (▲) from commercial pectic enzyme on HM-CL-AIS column. Chromatographic conditions: column, 2.5 cm × 20 cm; flow rate, 40 mL/h; and elution buffer, 0.01 M acetate buffer containing 0–1 M NaCl gradients. Fractions were collected and assayed for absorbance at 280 nm (—), concentration of NaCl (- - -), and each enzyme activity.

and interaction between pectin in HM-CL-AIS and enzymes in commercial liquid pectic enzyme, PE, PG, and PL might be separated by HM-CL-AIS; however, the activity chromatograms of PE, PG, and PL are difficult to separate on the HM-CL-AIS column eluted by buffers at pH 6−7 (data not shown).

PE activity fractions appear between fraction numbers 120 and 140 when the column is eluted at pH 4 (**Figure 1**). Meanwhile, it is shifted to a higher fraction number, ranging from 130 and 160, when the column is eluted at pH 5. This phenomenon is similar to the activity chromatograms of PG and PL on the HM-CL-AIS column eluted by buffers at pH 4−5.

The chromatograms of PG activity show that PG is eluted between fraction numbers 105 and 165 at pH 4 and shifts to a higher fraction number, ranging from 130 and 170 at pH 5. PL activity fractions appear in a wider fraction number range, between 120 and 200 at pH 4 and 140−220 at pH 5. Comparing the activity chromatograms of PE, PG, and PL eluted by buffers at pH 4−5 suggests that the HM-CL-AIS column is suitable for the separation of PL from PE and PG. Obviously, the use of the HM-CL-AIS column for PE and PG separation appears to be limited in the present study.

For extraction, clarification, and filtration improvement of fruit juice and wine, PL is more useful than PG in the food industries (*20*). PL is the only one capable of depolymerizing pectin without altering its esterification level (*21*) and cleaving the α-1, 4 bonds of highly esterified pectin without the previous demethylesterification by other enzymes (*22*). However, PG, the methyl ester groups of pectin, has to be previously hydrolyzed since PG prefers low methoxy pectin as a substrate and methanol is produced during the hydrolysis of methyl ester groups.

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**Table 1.** Separation Ability of PE, PG, and PL from Commercial Pectic Enzyme via HM-CL-AIS Chromatography

| fraction number | enzyme | total activity (U) | total protein (mg) | specific activity (mg/mL) | purification fold | yield (%) |
|---|---|---|---|---|---|---|
| | | | Commercial pectic enzyme | | | |
| | PE | 92.6 | 1.5 | 63.8 | 1.0 | 100.0 |
| | PG | 23.4 | 1.5 | 16.2 | 1.0 | 100.0 |
| | PL | 520.0 | 1.5 | 358.5 | 1.0 | 100.0 |
| | | | HM-CL-AIS column | | | |
| pH 4.0 | | | | | | |
| 97–128 | PE | 80.6 | 1.1 | 75.1 | 1.2 | 87.1 |
| | PG | 20.7 | 1.1 | 19.3 | 1.2 | 88.3 |
| 129–190 | PL | 440.2 | 0.2 | 2098.5 | 5.9 | 84.7 |
| pH 5.0 | | | | | | |
| 100–144 | PE | 71.7 | 1.0 | 70.7 | 1.1 | 77.4 |
| | PG | 20.1 | 1.0 | 19.9 | 1.2 | 85.9 |
| 145–200 | PL | 302.0 | 0.3 | 1094.2 | 3.1 | 58.2 |

**Table 2.** Physicochemical Properties of Wine Prepared from Grape Must in the Presence of Different Kinds of Pectic Enzymes[a]

| | grape must | control[b] | pectic enzyme | PE and PG | 1 PL | 3 PL |
|---|---|---|---|---|---|---|
| total soluble solids (°Brix) | 24.00 ± 0.00 a | 8.67 ± 0.23 b | 7.87 ± 0.42 d | 8.40 ± 0.00 b | 7.87 ± 0.12 c | 8.00 ± 0.00 c |
| specific gravity | 1.110 ± 0.000 a | 0.995 ± 0.001 c | 0.998 ± 0.000 b | 0.998 ± 0.000 b | 0.998 ± 0.000 b | 0.998 ± 0.000 b |
| ethanol (%) | 0.14 ± 0.01 b | 10.01 ± 0.02 a | 10.02 ± 0.05 a | 10.13 ± 0.27 a | 10.00 ± 0.11 a | 10.05 ± 0.15 a |
| pH | 3.02 ± 0.04 d | 3.23 ± 0.06 b,c | 3.21 ± 0.01 c | 3.26 ± 0.02 b,c | 3.29 ± 0.03 b | 3.35 ± 0.02 a |
| titratable acidity (%) | 1.85 ± 0.02 a | 1.67 ± 0.02 b | 1.67 ± 0.01 b | 1.59 ± 0.02 c | 1.67 ± 0.05 b | 1.56 ± 0.02 c |
| transmittance (% T) | 0.10 ± 0.00 e | 14.47 ± 0.86 d | 19.70 ± 0.80 b,c | 21.40 ± 4.35 b | 17.00 ± 1.83 c,d | *27.73 ± 3.10 a* |
| L | 0.56 ± 0.03 b | 0.23 ± 0.04 d | 0.28 ± 0.03 c,d | 0.35 ± 0.03 c | 0.55 ± 0.06 b | *0.68 ± 0.08 a* |
| A | 3.81 ± 0.10 b | 1.53 ± 0.25 d | 1.79 ± 0.12 c,d | 2.33 ± 0.38 c | 3.60 ± 0.44 b | *4.70 ± 0.60 a* |
| B | 0.90 ± 0.04 b | 0.30 ± 0.08 d | 0.43 ± 0.05 c,d | 1.14 ± 0.07 c | 0.85 ± 0.11 b | 1.11 ± 0.13 a |
| total pectin content[c] | 1.04 ± 0.13 a | 0.83 ± 0.05 b | 0.41 ± 0.05 c | 0.54 ± 0.11 c | 0.55 ± 0.05 c | 0.50 ± 0.05 c |

[a] Data bearing different letters in the same row are significantly different ($p < 0.05$). [b] Control group: without external enzyme adding. [c] g of anhydrogalacturonic acid/100 g of grape.

The recovery and purification of PE, PG, and PL from commercial pectic enzymes via the HM-CL-AIS column are higher at pH 4 (**Table 1**). When the HM-CL-AIS column was eluted at pH 4, the yield (%) and purification of PL were about 84.7% and 5.9, respectively. No PG activity was detected in the partial purified PL (fraction numbers 129–190); however, the isolated PL contains some residual PE activity (about 12% of the commercial pectic enzyme). On the basis of the previous results, the present procedures suggest an inexpensive, convenient, and effective methodology for high purification of PL from commercial pectic enzyme by an HM-CL-AIS column for industry use. The rest of the fractions (fraction numbers 97–128) containing PE and PG activities with 87–88% yield and 1.2 purification were also pooled and named as the PE and PG fraction for further use.

**Physicochemical Properties of Different Treatments.** The physicochemical properties of wine prepared from grape must are shown in **Table 2**. Ethanol content of the final wine products in each group was about 10%, suggesting that the addition of pectic enzyme, PE and PG, 1× PL, or 3× PL does not change the fermentation process. However, the decrease of total pectin content in all enzyme adding groups (0.41–0.55) is significant in comparison to the control (0.83).

The pH value of the 3× PL group (3.35 ± 0.02) was significantly higher than that in the other groups. It maybe due to the strong activity of PL (1060.8 units) adding to grape must for pectin depolymerization. It is interesting to find that the addition of the commercial pectic enzyme does not cause significant changes in the titratable acidity; however, the titratable acidity of the 3× PL group (1.56 ± 0.02%) and the PE and PG group (1.59 ± 0.02%) is significantly lower than the control (1.67 ± 0.02%). Faquembergue and Grassin (*23*) and Hohn (*24*) reported that the use of pectic enzymes in wine

preparation facilitated the formation of polygalacturonic acids and titratable acid. In this case, the variety and composition of pectin hydrolytic enzymes (i.e., lower PG activity and higher PL activity) will cause a decrease in titratable acidity.

The transmittance (% T) of wine in pectin hydrolytic enzyme adding groups (pectic enzyme, PE and PG, 1× PL, and 3× PL groups) is higher than the control. The 3× PL group (27.73 ± 3.10) is the highest among the groups. A decrease in cloud loss during fermentation was found in the presence of the PE inhibitor in carambola wine production (*3*). In this study, we discovered that cloud loss significantly increases in the pectic enzyme, PE and PG, and 3× PL groups of wine production.

In color, the values of lightness (*L* value), red content (*a* value), and yellow content (*b* value) increase in pectin hydrolytic enzyme adding groups, revealing that the increase in the pectin hydrolytic enzyme activity resulted in the increase of the brightness and red and yellow color of wine. These results are consistent with Èapounová and Drdák (*25*). They found that the maximum release of the red grape pigments took place within 4 or 5 days after the application of pectic enzyme preparations, while only 2–3 days was necessary to achieve the same effect in the control sample.

Moreover, comparing the 3× PL group to the 1× PL group, the great increase in PL activity induced an increase in the color characteristics. Comparing the 1× PL group to the pectic enzyme group, a decrease in PE (249.7 units) and PG (340.5 units) activities caused an increase in all color characteristics. When comparing the PE and PG group to the pectic enzyme group, the decrease in PL (371.8 units) and PG (157.5 units) activities caused an increase in the color characteristics. Wu et al. (*3*) also found that the loss of brightness is caused by a decrease in PE activity. That is, in the presence of pectin hydrolytic enzymes, the red grape pigments and aroma com-
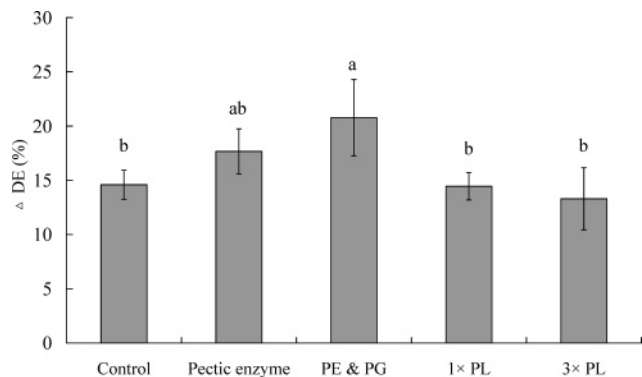
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**Figure 2.** Effect of different kinds of pectic enzymes on the difference in degree of esterification, $\rho$DE (%), of wine between day 0 and day 15 fermentation. Data bearing different superscripts are significantly different ($p < 0.05$). Control group: without external enzyme added; pectic enzyme group: with commercial pectic enzyme solution added; PE and PG group: with partial purified PE solution; 1× PL group: with partial purified PL solution; and 3× PL group: with 3-fold concentration partial purified PL solution of 1× PL group.
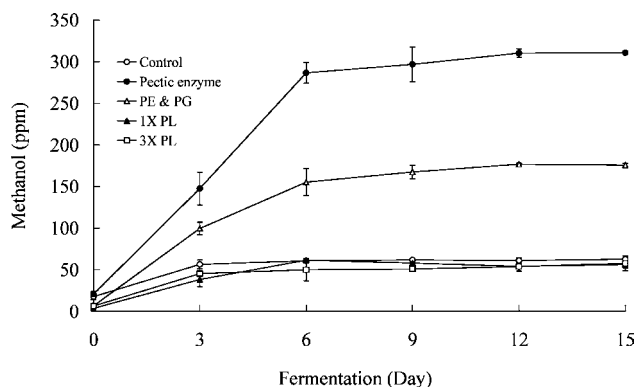


**Figure 3.** Changes in the methanol content of grape musts in the presence of different kinds of pectic enzymes during fermentation.

pounds are released faster due to the split of pectin. Meanwhile, the ratio of PE, PG, and PL in the reaction mixture may also be an important factor for their cooperation in pectin hydrolysis. The antioxidative activity of the wines was related to the total phenolics and total flavonoids (*26*), which are considered to be highly beneficial to health. Besides, the addition of pectin hydrolytic enzymes is good for releasing healthy compounds in wine products.

The DE of pectin in the starting material was about 74.0%, and it decreased to about 49.5% in the control and 48.4−60.2% in the pectin hydrolytic enzyme adding groups (data not shown). The difference in DE ($\rho$DE) of wine between day 0 and day 15 fermentation is shown in **Figure 2**. The $\rho$DE is 17.7 ± 2.1% in the pectic enzyme group, and it increases to the highest level of 20.8 ± 3.5% in the PE and PG group, while 93.0% of PL and 46.3% of PG activities in commercial pectic enzyme were removed. Comparing the 1× PL group to the pectic enzyme group, the elimination of PE and PG from the commercial pectic enzyme causes a decrease in $\rho$DE to 14.4 ± 1.3% during fermentation.

**Effect of Different Treatments on Methanol Formation.** The methanol content of grape must increased gradually to plateau at day 6 fermentation for pectic enzyme, PE and PG, and 1× PL groups (**Figure 3**). Changes in methanol content of the control (62.7 ppm) and PL adding groups (56.4−57.6 ppm) during fermentation are almost the same ($p > 0.05$). Higher level PE (263−265 units) results in a higher content of methanol

production in pectic enzyme and PE and PG groups. Methanol occurs naturally at a low level in the control group (62.7 ± 1.0 ppm) as was previously reported (*1−3*) and is mainly derived from pectin in the presence of intrinsic PE in fruit juice or must during fermentation.

Adding commercial pectic enzyme and partially purified PE and PG to grape must increases the methanol level from 62.7 ± 1.0 ppm (628 ± 13 mg/L of ethanol) in the control group to about 310.7 ± 1.5 ppm (3103 ± 16 mg/L of ethanol) in the pectic enzyme group and 175.8 ± 2.1 ppm (1736 ± 67 mg/L of ethanol) in the PE and PG group after 15 day fermentation. Nevertheless, the addition of PL to grape must does not cause an increase in methanol content. Hence, the difference of methanol contents between pectic enzyme and PE and PG groups reveals the fact that the decrease of methanol content (about 1367 mg/L of ethanol) might be caused by the decrease of PG activity (157.5 units). That is, the cooperation of PG with PE will greatly increase the production of methanol.

Pectin acts as a stabilizer of clouds and retards the speed of settling and filtration. Nevertheless, the clouds may result in an unpleasant taste of the red wine (*25*). This result strongly evidenced that virtually no methanol is produced in wine of the PL adding groups due to the elimination of PE activity from commercial pectic enzyme in this work. With the exception of methanol content, the use of partially purified PL for wine fermentation resulted in better physicochemical properties such as transmittance, lightness, redness, and lower total pectin content as that prepared in the control group.

In conclusion, the results present in this study are important for the wineries. It shows that the HM-CL-AIS column is a simple, inexpensive, convenient, and effective method for PL purification. The utilization of PL in winemaking allows an increase in red grape pigment release and a decrease in pectin content. More importantly, no extra methanol is produced by PL in wine production. According to our calculations, the cost for PL purification via HM-CL-AIS affinity gels including materials (<NT $2000) and payment for the time (NT $1600) is less than NT $3600. The cost will decrease in the large scale and industry levels when the quantity of PL production is amplified. Thus, this is an attractive way to reduce methanol production and improve the quality of the final product via replacing commercial pectic enzymes by partial purified PL in winemaking.

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