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# Comparative Analysis of Universal Protein Extraction Methodologies for Screening of Lipase Activity from Agricultural Products

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**Abstract:** Protein extraction techniques are absolutely required for the research of biological catalysts. The present study compared four universal protein extraction methodologies (ammonium sulfate precipitation, TCA/acetone precipitation, and two commercial kits) to provide practical information on protein extraction in order to discover a novel lipase in agricultural products. Yields of protein extraction from 24 domestic agricultural products and their specific activities were evaluated and compared with each other. TCA/acetone precipitation showed a relatively higher extraction yield (on average, 3.41 ± 1.08 mg protein/0.1 g sample) in crude protein extraction, whereas the Pierce™ Plant Total Protein Extraction Kit showed the highest specific lipase activity on average in both spectrophotometric (266.61 ± 235.78 μU/mg protein) and fluorometric (41.52 ± 32.63 μU/mg protein) assays. Our results suggest that commercial kits for the rapid extraction of soluble functional proteins would be a better choice than conventional precipitation techniques to perform the high-throughput screening of enzyme activity from plant sources. Finally, several agricultural products such as cordyceps, pepper, bracken, and hemp, all of which exhibited an excellent specific lipase activity, were proposed as promising candidates for a source of novel lipases.

**Keywords:** lipase; agricultural product; protein extraction; extraction yield; specific activity; spectrophotometric assay; fluorometric assay



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## 1. Introduction

Lipases (i.e., triacylglycerol hydrolase, EC 3.1.1.3) are a group of carboxylic-ester hydrolases that catalyze the hydrolysis (forward reaction) as well as the esterification (backward reaction) of triacylglycerol species as one of its major substrates [1]. These enzymes have unique substrate specificities (e.g., typoselectivity, regioselectivity, and stereoselectivity) [2] unlike other hydrolases, and on that basis, are able to catalyze specialized reactions suited to the needs of various industries such as the chemical, food, and pharmaceutical industries [3,4]. Hence, recent studies have continued to find novel lipases with rare properties from natural sources [1,5,6]. Most organisms from prokaryotes to eukaryotes are known to possess several inherent lipases, whereas almost all of the industrial lipases that have previously been discovered or developed have originated from bacteria or fungi [7,8]. In the case of higher plants such as agricultural and forest products, it is challenging to apply a top-down approach (i.e., from gene to protein) due to the lack of deciphered genome sequences to explore specific lipase genes and the practical difficulties of their heterologous expression for overproduction. Moreover, cultivation problems, complex tissue structures,

numerous phytochemicals, and the complicated regulation of genes and proteins cause bottom-up approaches (i.e., from activity to protein) to only generate a low yield of target proteins [9,10]. Therefore, to find novel and unique lipases in agricultural products, it is of primary importance to obtain a single protein with as much target catalytic activity as possible while using the appropriate protein extraction and purification methodologies to overcome these problems.

Extraction of a high amount of soluble protein from natural sources is one of the most crucial steps to isolate a target protein because the loss of proteins is an unavoidable occurrence during the purification process, although other endogenous proteins are present in a much greater quantity than the proteins of interest [11]. For that reason, numerous methodologies based on ammonium sulfate ($(NH_4)_2SO_4$) precipitation or trichloroacetic acid (TCA)/acetone precipitation have been developed and performed to effectively extract total proteins at each experimental condition [12,13]. First, a methodology using ammonium sulfate precipitation called salting out is the most commonly used technique for protein extraction from a solution and is conducted by lowering the solubility of proteins through competing solubility in water with more soluble neutral salts [13]. Each protein precipitates at a specific concentration of ammonium sulfate, meaning that the desired proteins can be roughly fractionated from other proteins in the total protein extraction step. Second, instead of using salts, TCA/acetone precipitation is also a universally used technique to purify and concentrate total proteins by lowering the pH value of a solution, which promotes isoelectric precipitation as well as the disruption of the hydrogen bonding of the protein with water molecules [11]. Third, several commercial kits using other chaotropic salts and protease inhibitor cocktails for total protein extraction from various sources have recently become available at the laboratory scale. Unfortunately, however, protein extractions in previous studies on plant-derived enzymes have been conducted sporadically, and furthermore, there is no comprehensive or comparative information on practical protein extraction from agricultural products.

In this study, we applied four universal protein extraction methodologies—ammonium sulfate precipitation, TCA/acetone precipitation, and two commercial kits—to several domestic agricultural products to reveal a global tendency and provide more practical information regarding protein extraction to find novel lipases from plant samples. Protein yields of crude protein extracts were evaluated and compared with their specific activities. The catalytic activity of the lipases was measured by both spectrophotometric and fluorometric assays using *p*-nitrophenyl palmitate (*p*-NPP) [14] and 4-methylumbelliferyl oleate (4-MUO) [15], respectively. The relationship between protein extraction yield and specific activity according to the applied extraction methodology and lipase assay is discussed, and additionally, several promising candidates for lipase can be proposed. The following results can provide practical information regarding the extraction of total proteins and the screening of functional proteins from agricultural products.

## 2. Results and Discussion

### *2.1. Comparative Analysis of Protein Extraction Efficiency*

To specifically compare the protein extraction efficiency between the difference extraction methodologies, we were first required to select appropriate agricultural product candidates with lipase activity catalytic activity. From the 9th revision of Korean National Standard Food Composition Table [16], 87 kinds of domestic agricultural products containing both more than 10.0 g of different proteins and 1.0 g of lipids were chosen, and their crude protein extracts were screened for lipase activity using both spectrophotometric and fluorometric assays (Supplementary Data S1). Protein extraction in this step was performed using the Pierce™ Plant Total Protein Extraction Kit because it is designed to rapidly extract a qualitative sample of proteins from any type of plant tissue without any type of precipitation process. During the reaction, significant increases of raw absorbance (converted into *p*-nitrophenol, $\geq$0.01 mM) or fluorescence (converted into 4-methylumbelliferone, $\geq$0.01 mM) values, with the exception of background signals, were observed in 58 and

27 different crude protein extracts, respectively, which indicated that those crude protein extracts possess putative enzymes with lipolytic activity at certain levels. Our investigation began with 24 different domestic agricultural products concurrently exhibiting potential lipase activity in both assays, and these final candidates were categorized into seven classifications: cereals, potatoes, peas, vegetables, mushrooms, nuts, and seeds (Supplementary Data S2).

Crude protein extracts from 24 different of domestic agricultural products were freshly obtained using four universal protein extraction methodologies. After all of isolation processes were complete, which are briefly represented in Figure 1, the total protein amounts and converted yields of protein extraction (mg of total protein per 0.1 g of sample) were quantified and compared among the methodologies (Table 1 and Figure 2). Overall, TCA/acetone precipitation showed a relatively higher extraction yield (on average, 3.41 ± 1.08 mg protein/0.1 g sample) in the crude protein extraction of agricultural products than the other methodologies, with several exceptions for peas and seeds. During TCA/acetone precipitation, the samples were completely pulverized, and interfering compounds (generally water-insoluble) were repeatedly rinsed out, leaving almost only the whole proteins in the precipitate [12,17]. These precipitated proteins could be retrieved directly using a buffer of choice without any loss incurred by processes such as column binding, desalting, or filtration, which was negatively correlated with extraction efficiency. By contrast, ammonium sulfate precipitation, which is another commonly used protein precipitation technique, showed a much lower protein extraction yield (on average, 0.20 ± 0.078 mg protein/0.1 g sample) in all of the samples, probably due to the inevitable desalting process for the removal of ammonium salts [18,19]. Nevertheless, ammonium sulfate precipitation was the only method able to extract crude proteins, unlike TCA/acetone precipitation or the commercial kits (0.1 g sample). Starting with a 50 g sample, the absolute amounts of total protein extracted using ammonium sulfate precipitation were approximately 10 to 250 times higher than those using other methodologies. These results indicated that ammonium sulfate precipitation would be efficient when the mass production of proteins is required despite its low extraction efficiency.

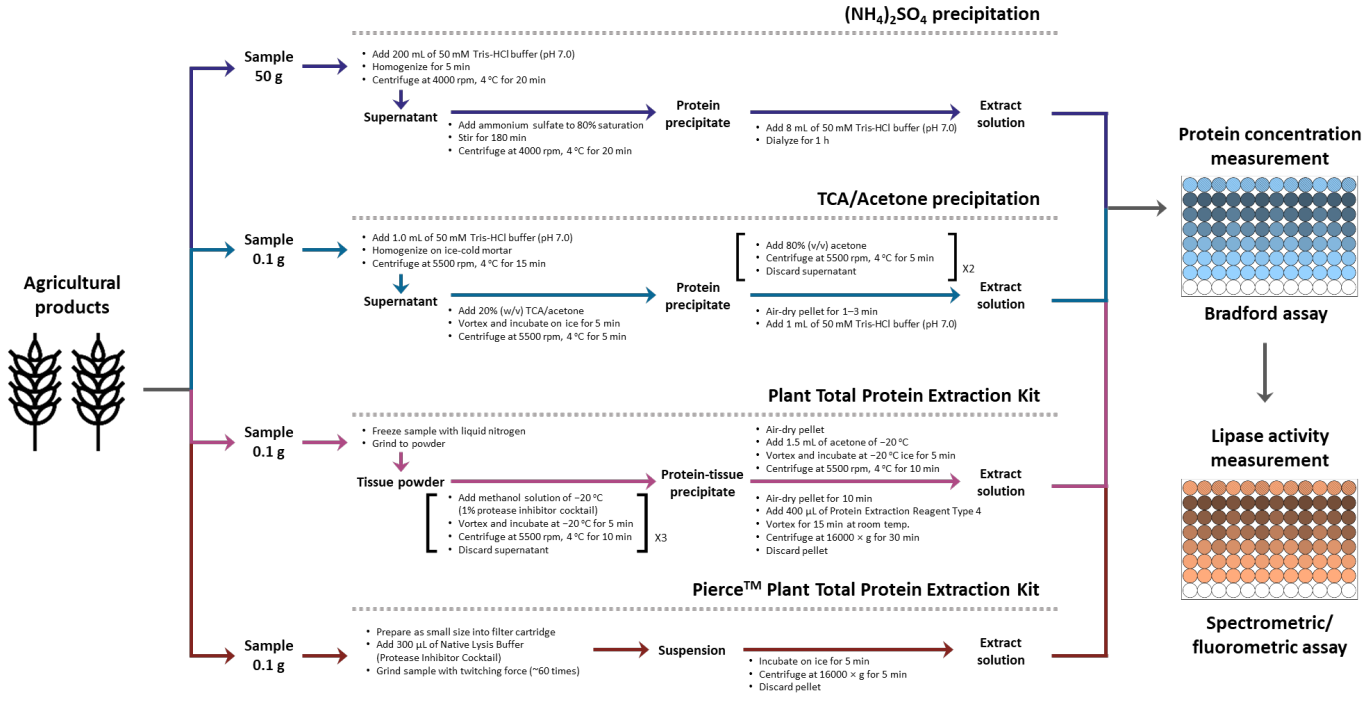


**Figure 1.** Schematic diagram representing workflow of protein extraction methodologies and the high-throughput screening of lipase activity.

**Table 1.** Protein extraction yield of each protein extraction methodology from 24 domestic agricultural products.

| Source [1] | $(NH_4)_2SO_4$ Precipitation | | TCA/Acetone Precipitation | | Commercial Kit A [2] | | Commercial Kit B [3] | |
|---|---|---|---|---|---|---|---|---|
| | Concentration (mg/mL) | Extraction Yield (mg/0.1 g Sample) | Concentration (mg/mL) | Extraction Yield (mg/0.1 g Sample) | Concentration (mg/mL) | Extraction Yield (mg/0.1 g Sample) | Concentration (mg/mL) | Extraction Yield (mg/0.1 g Sample) |
| 1 | 14.05 | 0.22 | 2.75 | 2.75 | 4.77 | 1.91 | 3.06 | 0.92 |
| 2 | 13.93 | 0.22 | 4.56 | 4.56 | 4.56 | 1.83 | 12.99 | 3.90 |
| 3 | 16.36 | 0.26 | 2.94 | 2.94 | 4.01 | 1.60 | 8.74 | 2.62 |
| 4 | 4.25 | 0.07 | 4.40 | 4.40 | 11.02 | 4.41 | 13.72 | 4.11 |
| 5 | 13.45 | 0.22 | 2.84 | 2.84 | 14.28 | 5.71 | 8.34 | 2.50 |
| 6 | 13.26 | 0.21 | 3.01 | 3.01 | 12.99 | 5.19 | 11.39 | 3.42 |
| 7 | 14.78 | 0.24 | 3.01 | 3.01 | 4.62 | 1.85 | 7.30 | 2.19 |
| 8 | 16.38 | 0.26 | 2.87 | 2.87 | 5.74 | 2.30 | 7.09 | 2.13 |
| 9 | 16.74 | 0.27 | 2.95 | 2.95 | 5.03 | 2.01 | 7.01 | 2.10 |
| 10 | 13.74 | 0.22 | 2.79 | 2.79 | 2.79 | 1.12 | 9.22 | 2.77 |
| 11 | 15.63 | 0.25 | 3.80 | 3.80 | 14.07 | 5.63 | 14.78 | 4.44 |
| 12 | 18.47 | 0.30 | 3.82 | 3.82 | 11.25 | 4.50 | 15.20 | 4.56 |
| 13 | 18.63 | 0.30 | 3.19 | 3.19 | 9.12 | 3.65 | 13.65 | 4.10 |
| 14 | 16.02 | 0.26 | 6.78 | 6.78 | 13.02 | 5.21 | 14.58 | 4.37 |
| 15 | 15.34 | 0.25 | 4.34 | 4.34 | 11.32 | 4.53 | 16.89 | 5.07 |
| 16 | 8.75 | 0.14 | 2.87 | 2.87 | 5.25 | 2.10 | 4.52 | 1.36 |
| 17 | 9.92 | 0.16 | 2.83 | 2.83 | 2.83 | 1.13 | 5.17 | 1.55 |
| 18 | 4.19 | 0.07 | 2.79 | 2.79 | 2.79 | 1.12 | 3.47 | 1.04 |
| 19 | 12.85 | 0.21 | 2.76 | 2.76 | 2.76 | 1.11 | 5.23 | 1.57 |
| 20 | 4.24 | 0.07 | 2.71 | 2.71 | 3.37 | 1.35 | 4.27 | 1.28 |
| 21 | 5.47 | 0.09 | 5.89 | 5.89 | 5.84 | 2.34 | 5.64 | 1.69 |
| 22 | 15.45 | 0.25 | 2.69 | 2.69 | 2.69 | 1.07 | 5.08 | 1.52 |
| 23 | 3.41 | 0.05 | 2.54 | 2.54 | 3.77 | 1.51 | 4.36 | 1.31 |
| 24 | 8.68 | 0.14 | 2.82 | 2.82 | 4.59 | 1.84 | 5.50 | 1.65 |

[1] 24 final candidates described in Supplementary Data S2. [2] Plant Total Protein Extraction Kit (Sigma-Aldrich Co.). [3] Pierce™ Plant Total Protein Extraction Kit (Thermo Fisher Scientific Co.).

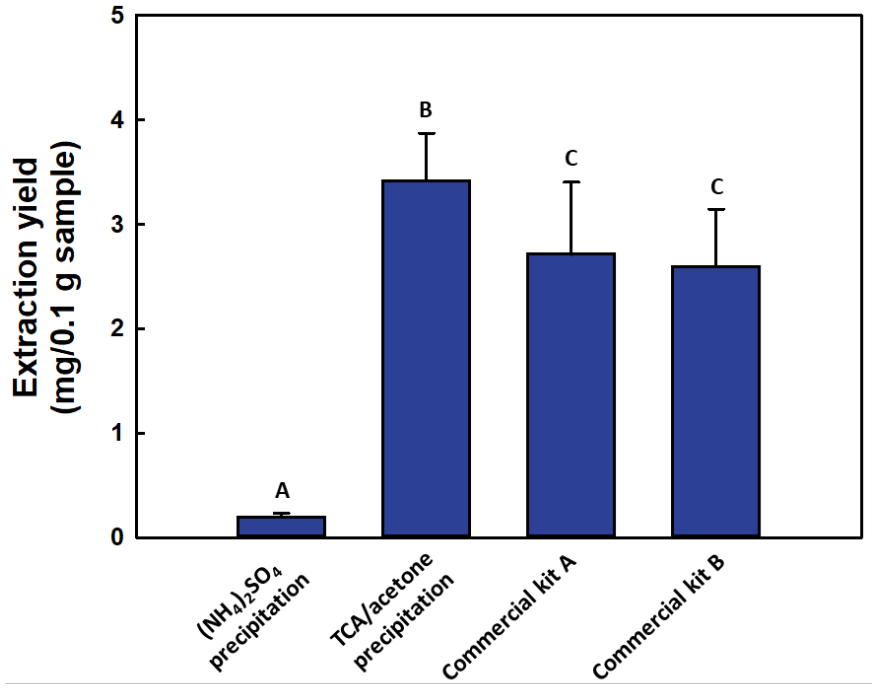


**Figure 2.** Efficiency comparison of protein extraction yield of universal protein extraction methodologies from 24 Korean domestic agricultural products. Means with different superscripts differ significantly ($p < 0.05$).

In the case of the commercial kits, the Plant Total Protein Extraction Kit showed a slightly higher extraction yield (on average, 2.71 ± 1.64 mg protein/0.1 g sample) than that of the Pierce™ Plant Total Protein Extraction Kit (on average, 2.59 ± 1.31 mg protein/0.1 g sample). The use of commercial kits, especially the Pierce™ Plant Total Protein Extraction Kit, simplified the process of protein extraction and overcame several drawbacks of conventional protein precipitation techniques such as time-consuming and laborious work [12,13]. Indeed, with the exception of ammonium sulfate precipitation, it was considered that

there was no substantial difference in extraction yield between these methodologies. These methodologies are already well-optimized by previous researchers to extract proteins in lab-scale experiment, and we conducted all experiments in the optimized conditions. While it is important to obtain the highest possible amount of proteins, it is also crucial that proteins exist in their intact form and maintain their functions. Therefore, it was more critical to measure the apparent specific activity (i.e., the catalytic activity per quantity of protein) of each extracted protein to compare practical performance to the high-throughput lipase screening in agricultural products.

### *2.2. Comparative Analysis of Catalytic Activity by Spectrophotometric Assay*

To evaluate the specific activity of protein extracts, we performed a high-throughput screening of lipase activity in standard reaction conditions (pH 7.0 and 30 °C) using spectrophotometric assay that employs *p*-NPP as an alternative substrate. The hydrolysis of *p*-NPP showed constant linearity at the early stages of the reaction (data not shown), and catalytic activity (expressed in velocity, nM/min) was determined from the non-linear regression curves of *p*-nitrophenol concentration versus reaction time. Based on the reaction data for the protein concentrations, the apparent specific activity (μU/mg protein) of the protein extracts was evaluated and compared with each other (Table 2 and Figure 3A). Surprisingly, protein extracts extracted using TCA/acetone precipitation exhibited the lowest level of specific lipase activity (up to maximum mean of 3300%), whereas its protein extraction yield was relatively higher than that of the other extraction methods. Generally, the combination of TCA and acetone chemicals to precipitate proteins is an efficient strategy to extract the total proteins from any type of sample, in other words, to execute indiscriminate protein extraction. It is known that TCA/acetone precipitation is more effective than either TCA or acetone alone, somewhat minimizing protein denaturation and protease activity as well as reducing other contaminants such as salts or polyphenols [12]. However, owing to the unavoidable exposure to the low pH solution (i.e., TCA) or organic solvent (i.e., acetone) during the extraction process, the extended disruption or modification of the protein molecules might be provoked [20], resulting in the loss of catalytic activity. Moreover, in the crude protein extract of natural sources, numerous endogenous proteins including most structural and functional proteins are present, along with a small proportion of the enzymes of interest. Both of these types of proteins can be totally extracted through TCA/acetone precipitation, and these disadvantageous circumstances would impair enzymes, causing them to be hardly detected during the high-throughput screening, as shown in this study. Therefore, instead of using this method for high-throughput screening, TCA/acetone precipitation can be the preferred method used in proteomics [21] or heterologous expression [22,23], which requires a high yield of total protein extraction.

On the other hand, both of the commercial kits showed relatively higher specific activities than those of the conventional methodologies in the spectrophotometric screening of lipase activity. As aforementioned, it was crucial that more soluble functional proteins of interest were maximally extracted than other endogenous proteins in order to detect enzyme activity more specifically. These kits were based on different operating principles, and they both introduced filtration processes to remove other interfering substances using a specific column and protease-inhibiting additive to mitigate constitutional protease activity, resulting in improvement in extraction yield as well as specific activity. The Plant Total Protein Extraction Kit is based on the same operating principle as the precipitation methodologies. Notably, unlike the other kit, the Pierce™ Plant Total Protein Extraction Kit adopted a native lysis procedure to directly obtain proteins in a soluble form, without any precipitation or regeneration of the proteins. This strategy allowed the functional proteins (in this case, enzymes possessing lipase activity) to be isolated with minimal loss of catalytic activity and also enabled efficient and rapid protein extraction to be performed in less time than the other methodologies. The ranking of specific activity between the agricultural products was partially inconsistent according to the extraction methodologies; however, it was evident that both commercial kits are suitable for performing the high-throughput

screening of lipase activity in agricultural products based on the spectrophotometric assay. In addition, some agricultural products such as bracken, cordyceps, and the seeds of red pepper exhibited an excellent specific lipase activity. Several GDSL-type lipases from pepper (**21**, conspecific, *Capsicum annuum*) [24] and *sn*-1(3) regioselective lipases from cordyceps (**16**, *Cordyceps militaris*) [1,25] were previously reported, while there was no reported information on the lipases in bracken (**24**, *Pteridium latiusculum*). Therefore, these results suggest the availability of these agricultural products as promising candidates as a source of novel lipases.

**Table 2.** Specific activity of crude protein extracts from 24 domestic agricultural products (spectrophotometric assay).

| Source [1] | $(NH_4)_2SO_4$ Precipitation | | TCA/Acetone Precipitation | | Commercial Kit A [2] | | Commercial Kit B [3] | |
|---|---|---|---|---|---|---|---|---|
| | Velocity (nM/min) | Specific Activity (μU/mg) | Velocity (nM/min) | Specific Activity (μU/mg) | Velocity (nM/min) | Specific Activity (μU/mg) | Velocity (nM/min) | Specific Activity (μU/mg) |
| 1 | 864.55 | 61.55 | 62.12 | 22.56 | 1518.92 | 318.37 | 2318.93 | 757.52 |
| 2 | 923.22 | 66.26 | 57.29 | 12.56 | 445.22 | 97.57 | 2018.32 | 155.39 |
| 3 | 406.91 | 24.88 | 23.12 | 7.87 | 377.57 | 94.20 | 2213.32 | 253.15 |
| 4 | 129.08 | 30.39 | 81.11 | 18.45 | 2607.46 | 236.60 | 2626.09 | 191.46 |
| 5 | 589.83 | 43.87 | 82.83 | 29.18 | 583.61 | 40.87 | 1522.71 | 182.49 |
| 6 | 202.94 | 15.30 | 45.56 | 15.13 | 1284.57 | 98.93 | 963.60 | 84.58 |
| 7 | 168.08 | 11.38 | 47.97 | 15.92 | 670.24 | 145.11 | 607.43 | 83.25 |
| 8 | 311.65 | 19.02 | 43.49 | 15.17 | 698.54 | 121.63 | 608.12 | 85.73 |
| 9 | 708.90 | 42.34 | 21.05 | 7.15 | 594.66 | 118.32 | 1236.60 | 176.33 |
| 10 | 527.70 | 38.41 | 61.78 | 22.13 | 446.25 | 159.87 | 980.17 | 106.29 |
| 11 | 129.08 | 8.26 | 183.95 | 48.39 | 1503.73 | 106.89 | 1348.08 | 91.19 |
| 12 | 1893.73 | 102.52 | 86.97 | 22.77 | 901.82 | 80.18 | 1492.69 | 98.20 |
| 13 | 2400.38 | 128.87 | 62.81 | 19.66 | 427.62 | 46.88 | 1175.17 | 86.09 |
| 14 | 1221.76 | 76.28 | 91.11 | 13.44 | 923.57 | 70.92 | 3819.90 | 262.08 |
| 15 | 164.97 | 10.75 | 81.80 | 18.85 | 1151.35 | 101.73 | 2042.13 | 120.93 |
| 16 | 64.88 | 7.41 | 173.60 | 60.43 | 1168.61 | 222.65 | 3749.84 | 829.36 |
| 17 | 1648.34 | 166.10 | 81.11 | 28.68 | 1752.91 | 619.96 | 3276.66 | 633.42 |
| 18 | 1336.34 | 318.78 | 65.23 | 23.38 | 2224.36 | 797.43 | 1339.45 | 386.08 |
| 19 | 810.02 | 63.03 | 57.98 | 20.99 | 720.63 | 260.86 | 955.66 | 182.80 |
| 20 | 503.20 | 118.67 | 59.71 | 22.05 | 1204.85 | 357.61 | 517.00 | 121.00 |
| 21 | 1757.75 | 321.17 | 155.65 | 26.41 | 5668.41 | 970.45 | 1941.36 | 344.09 |
| 22 | 143.92 | 9.32 | 76.27 | 28.39 | 868.00 | 323.10 | 1653.86 | 325.37 |
| 23 | 272.65 | 79.93 | 67.30 | 26.48 | 1158.95 | 307.72 | 404.84 | 92.87 |
| 24 | 261.26 | 30.11 | 62.12 | 22.05 | 5075.13 | 1106.15 | 4119.47 | 748.99 |

[1] 24 final candidates described in Supplementary Data S2. [2] Plant Total Protein Extraction Kit (Sigma-Aldrich Co.). [3] Pierce™ Plant Total Protein Extraction Kit (Thermo Fisher Scientific Co.).

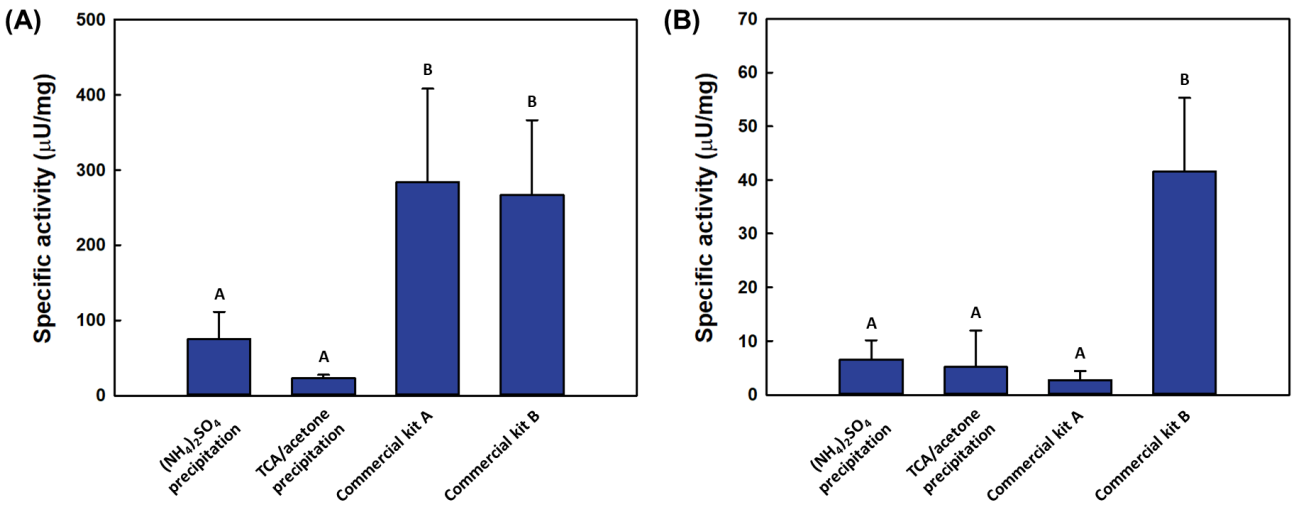


**Figure 3.** Detected specific activity of the extracts from 24 Korean domestic agricultural products according to the protein extraction methodologies. (**A**) Spectrophotometric assay. (**B**) Fluorometric assay. Means with different superscripts differ significantly ($p < 0.05$).

### *2.3. Comparative Analysis of Catalytic Activity by Fluorometric Assay*

Generally, agricultural products contain a large number of colored phytochemicals (i.e., primary or secondary metabolites) such as polyphenols and carotenoids [26]. The complete removal of colored compounds from plants is somewhat complicated, and a certain amount of these remnants in crude protein extract can interfere with the detection of enzyme activity in direct as well as indirect ways. Our spectrophotometric assay was based on application of *p*-NPP as a substrate, which liberates yellow-colored *p*-nitrophenol through lipase activity in an aqueous solution (pH 7.0) at room temperature [27]. The coexistence of more than one colored compound in the reaction medium may affect or conceal the raw value of absorbance of *p*-nitrophenol, which eventually confuses the reaction outcomes. For that reason, another substrate should be used for high-throughput screening to discover novel lipases from natural sources. In this study, we also performed a fluorescence assay using 4-MUO as an alternative substrate, which detects 4-methylumbelliferone. The specific activity of each protein extract was evaluated at standard reaction conditions (pH 7.0 and 30 °C). The reaction showed constant linearity at the early stages of the reaction (data not shown), and catalytic activity was determined from the non-linear regression curves of 4-methylumbelliferone concentration versus reaction time. In the same manner as the spectrophotometric assay, the apparent specific activity of each protein extract was examined (Table 3 and Figure 3B).

**Table 3.** Specific activity of crude protein extracts from 24 domestic agricultural products (fluorometric assay).

| Source [1] | $(NH_4)_2SO_4$ Precipitation | | TCA/Acetone Precipitation | | Commercial Kit A [2] | | Commercial Kit B [3] | |
|---|---|---|---|---|---|---|---|---|
| | Velocity (nM/min) | Specific Activity (μU/mg) | Velocity (nM/min) | Specific Activity (μU/mg) | Velocity (nM/min) | Specific Activity (μU/mg) | Velocity (nM/min) | Specific Activity (μU/mg) |
| 1 | 361.51 | 25.74 | 1.50 | 0.54 | 7.17 | 1.50 | 378.94 | 123.79 |
| 2 | 30.01 | 2.15 | 6.34 | 1.39 | 6.34 | 1.39 | 235.61 | 18.14 |
| 3 | 23.07 | 1.41 | 6.90 | 2.35 | 6.81 | 1.70 | 203.06 | 23.23 |
| 4 | 120.32 | 28.33 | 1.46 | 0.33 | 7.73 | 0.70 | 301.71 | 22.00 |
| 5 | 56.34 | 4.19 | 7.83 | 2.76 | 1.05 | 0.07 | 334.82 | 40.13 |
| 6 | 44.51 | 3.36 | 6.04 | 2.01 | 5.69 | 0.44 | 351.70 | 30.87 |
| 7 | 3.36 | 0.23 | 9.11 | 3.02 | 19.48 | 4.22 | 136.49 | 18.71 |
| 8 | 5.03 | 0.31 | 7.02 | 2.45 | 6.10 | 1.06 | 196.31 | 27.67 |
| 9 | 47.76 | 2.85 | 30.09 | 10.21 | 10.61 | 2.11 | 193.99 | 27.66 |
| 10 | 60.78 | 4.42 | 19.29 | 6.91 | 19.29 | 6.91 | 195.80 | 21.23 |
| 11 | 52.10 | 3.33 | 0.88 | 0.23 | 23.25 | 1.65 | 170.50 | 11.53 |
| 12 | 73.24 | 3.96 | 2.15 | 0.56 | 17.09 | 1.52 | 256.05 | 16.85 |
| 13 | 23.29 | 1.25 | 1.55 | 0.49 | 6.39 | 0.70 | 128.78 | 9.43 |
| 14 | 73.38 | 4.58 | 0.37 | 0.05 | 19.19 | 1.47 | 200.25 | 13.74 |
| 15 | 5.96 | 0.39 | 1.71 | 0.39 | 17.37 | 1.53 | 165.99 | 9.83 |
| 16 | 24.29 | 2.77 | 231.52 | 80.59 | 4.25 | 0.81 | 501.09 | 110.83 |
| 17 | 156.46 | 15.77 | 5.19 | 1.84 | 5.19 | 1.84 | 457.28 | 88.40 |
| 18 | 34.87 | 8.32 | 3.74 | 1.34 | 3.74 | 1.34 | 228.32 | 65.81 |
| 19 | 43.64 | 3.40 | 5.82 | 2.11 | 5.82 | 2.11 | 139.47 | 26.68 |
| 20 | 107.74 | 25.41 | 2.69 | 0.99 | 15.56 | 4.62 | 208.58 | 48.82 |
| 21 | 39.61 | 7.24 | 3.14 | 0.53 | 0.57 | 0.10 | 462.33 | 81.95 |
| 22 | 3.60 | 0.23 | 4.40 | 1.64 | 4.40 | 1.64 | 383.47 | 75.44 |
| 23 | 28.23 | 8.28 | 1.80 | 0.71 | 20.81 | 5.52 | 208.42 | 47.81 |
| 24 | 0.17 | 0.02 | 0.97 | 0.34 | 91.75 | 20.00 | 197.34 | 35.88 |

[1] 24 final candidates described in Supplementary Data S2. [2] Plant Total Protein Extraction Kit (Sigma-Aldrich Co.). [3] Pierce™ Plant Total Protein Extraction Kit (Thermo Fisher Scientific Co.).

Crude proteins extracted using the Pierce™ Plant Total Protein Extraction Kit showed the highest specific lipase activity on average (41.52 ± 32.63 μU/mg protein), and its extraction tendency was highly similar to that of the spectrophotometric assay. In other words, the putative lipase activity detected from both lipase assays was cross-checked and adequately validated. Unfortunately, other methodologies based on protein precipitation, including the Plant Total Protein Extraction Kit, only showed a low level of specific activity. In the standardized conditions of 100% total proteins, the results should theoretically be identical in both spectrophotometric and fluorometric assays, and the precipitated

proteins should be totally soluble [28]; however, fluorescence-interfering substances such as remnants of precipitated proteins and other chemicals could remain in rehydrated precipitant solution. Consequently, these results eventually suggested that the Pierce™ Plant Total Protein Extraction Kit could be the best choice for the lipase screening of agricultural products in both assays. In the fluorescence assay, we also found several candidates for the source of lipases, such as potato (**1**, *Solanum tuberosum*) and hemp (**4**, *Cannabis sativa*) seed, which exhibited excellent specific lipase activity against 4-MUO. There was no reported information regarding lipase in hemp, whereas potatoes are already known to produce several lipases, for example, patatin, a family of glycoproteins having lipase activity to cleave fatty acids in membrane lipids [29,30].

Protein extraction techniques are absolutely required for various types of protein research. Considering the extraction yield as well as specific activity compared in this study, each protein extraction methodology should be selected based on a particular use or the purpose of the experiment. Ammonium sulfate precipitation is still most suitable for mass protein extraction, and various modified protocols of TCA/acetone precipitation are an excellent choice to conduct proteomics or other protein studies at a small scale. Nevertheless, to perform high-throughput screening of enzyme activity from plant sources, commercial kits for the rapid extraction of soluble functional proteins would be a better choice than conventional precipitation techniques based on our results. In addition, additional modifications of these methodologies using additives such as surfactants and chaotropic agents could enhance the extraction yield according to several previous studies, which optimized extraction methodologies for proteins in accordance with their specific purpose [31,32]. Of course, after the preliminary screening of promising candidates for an enzyme of interest, further purification, decisive activity assay, and genetic analysis should be completed to validate those results.

## 3. Materials and Methods

### *3.1. Materials*

Ammonium sulfate (≥99.5%, ACS reagent grade), trichloroacetic acid (≥99.0%, ACS reagent grade), and the Plant Total Protein Extraction Kit (suitable for any plant species or tissue) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). the Pierce™ Plant Total Protein Extraction Kit was purchased from Thermo Fisher Scientific Co. (Rockford, IL, USA). The Bio-Rad Protein Assay Kit II (including bovine serum albumin as a standard compound) for the Bradford assay was purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Substrates for the lipase assay, *p*-nitrophenyl palmitate (*p*-NPP, ≥98.0%), and 4-methylumbelliferyl oleate (4-MUO, ≥95.0%), were purchased from Sigma-Aldrich Co., and the reaction products, *p*-nitrophenol (≥99.0%) and 4-methylumbelliferone (≥98.0%), were purchased from Sigma-Aldrich Co. as well. All other chemicals were of guaranteed reagent grade and were used after filtration through a membrane filter (0.45 μm). The domestic agricultural products were purchased from local and open markets in the Republic of Korea. Before use, the seeds were soaked in water and covered with a layer of wet cotton to germinate at ambient temperature in the dark. The Costar® 96-well microplates (black wall, clear bottom) used for the fluorometric assay were purchased from Corning Co. (Corning, NY, USA). The JetBiofil® 96-well microplates (clear wall, clear bottom) used for the spectrophotometric assay were purchased from Guangzhou Jet Bio-Filtration Co. (Guangzhou, China).

### *3.2. Protein Extraction from Agricultural Products*

#### 3.2.1. Ammonium Sulfate [$(NH_4)_2SO_4$] Precipitation

The crude protein extracts were fractionated via ammonium sulfate precipitation at 80% saturation as previously described [1] with slight modifications. First, 50 g of ground agricultural products were dissolved in 200 mL of 50 mM Tris-HCl buffer (pH 7.0), homogenized for 5 min, and centrifugated at 4000 rpm for 20 min at refrigeration temperature. Solid amounts (gram) of ammonium sulfate were added to 100 mL of the supernatant solutions to

reach the desired saturation percent at 4 °C, as calculated using Ammonium Sulfate Calculator (accessed on 10 February 2021, https://www.encorbio.com/protocols/AM-SO4.htm, EnCor Biotechnology Inc., Gainesville, FL, USA). The samples were continuously stirred for 180 min to ensure perfect dissolution of the added ammonium sulfates and centrifugated at 4000 rpm for 20 min at 4 °C to pellet the precipitated proteins. The supernatant solutions were discarded, and the remnants were resuspended in 8 mL of 50 mM Tris-HCl buffer (pH 7.0). The suspensions were then dialyzed for 1 h using dialysis sacks (35 mm, MWCO 12 kDa, Sigma-Aldrich Co.) for further analysis. The concentrations of extracted protein in the samples were measured in 96-well microplates by means of Bradford assay [33] and using bovine serum albumin as a standard (Supplementary Data S3).

#### 3.2.2. TCA/Acetone Precipitation

The crude protein extracts were fractionated via modified TCA/acetone precipitation as previously described [12] with slight modifications. First, 100 mg of fresh agricultural products were dissolved in 1.0 mL of 50 mM Tris-HCl buffer (pH 7.0), homogenized in pre-cooled mortar on ice, and centrifugated at 5500 rpm for 15 min at refrigeration temperature. 20% (*w*/*v*) TCA/acetone was then added to the supernatants (1:1, *v*/*v*) with vigorous vortexing, and the mixtures were placed on ice for 5 min before centrifugation at 5500 rpm for 5 min at 4 °C to pellet the precipitated proteins. The supernatant solutions were discarded, and the pellets were washed with 80% (*v*/*v*) acetone and centrifuged as above. The washing process was repeated once. Finally, the protein precipitates were air-dried for a short time (1–3 min) and resuspended in 1 mL of 50 mM Tris-HCl buffer (pH 7.0) for further analysis. The final concentrations of the extracted protein in the samples were measured in 96-well microplates by means of Bradford assay as aforementioned.

#### 3.2.3. Protein Extraction Using Plant Total Protein Extraction Kit

The Plant Total Protein Extraction Kit (Sigma-Aldrich Co.) was employed according to the manufacturer's instructions. One hundred milligrams of the samples that had been frozen in liquid nitrogen ($N_2$) were ground to a fine powder without any thawing. The ground samples were transferred to V-bottom freezing vials (2 mL), and pre-cooled (−20 °C) methanol solution (containing 1% (*v*/*v*) Protease Inhibitor Cocktail) was immediately added to the samples through vigorous vortexing. The samples were then incubated for 5 min at −20 °C with periodic vortexing before centrifugation at 5500 rpm for 10 min at 4 °C to pellet the proteins and plant debris. The supernatants were discarded, and the methanol extraction step was repeated twice more. After the air-drying of the methanol in the pellets, 1.5 mL of pre-cooled (−20 °C) acetone was added through vigorous vortexing, placed for 5 min at −20 °C, and centrifugated at 5500 rpm for 10 min at 4 °C. The supernatants were discarded, and the remnants were allowed to be air-dried for 10 min before re-suspension in 400 µL of Protein Extraction Reagent Type 4 with vigorous vortexing for 15 min at room temperature. Finally, the suspensions were centrifugated at 16,000 × *g* for 30 min to pellet only the plant tissue debris. The final concentrations of extracted protein in the samples were measured in 96-well microplates by means of Bradford assay as aforementioned.

#### 3.2.4. Protein Extraction Using Pierce™ Plant Total Protein Extraction Kit

The Pierce™ Plant Total Protein Extraction Kit (Thermo Fisher Scientific Co.) was employed according to the manufacturer's instructions. First, the plant samples (100 mg) were manipulated into a smaller size through folding, punching, cutting, or grinding and placed into the filter cartridge. After that, 300 µL of Native Lysis Buffer (with Thermo Scientific™ Halt Protease Inhibitor Cocktail) was added as a filter to obtain the native form of the total protein extracts, and the samples were ground with a plastic rod approximately 60 times using twisting force. The samples were incubated for 5 min on ice before centrifugation at 16,000× *g* for 5 min to pellet any insoluble materials except for the native total proteins. Finally, the supernatants were transferred to a new tube and used for further

analysis. The final concentrations of the extracted protein in the samples were measured in 96-well microplates by means of Bradford protein assay as aforementioned. All protein extract samples were stored at refrigeration temperature until use.

### 3.3. Spectrophotometric Assay for Measuring Lipase Activity

The spectrophotometric assay for measuring the catalytic activity of lipase was established using *p*-NPP as an alternative substrate as previously described [1]. The substrate solution (5.00 mM) was prepared by dissolving 62.29 mg of *p*-NPP into 33.0 mL of distilled water containing 11.0 mg of sodium dodecyl sulfate and 330.0 mg of Triton X-100. The solution was incubated at 65 °C for 20 min with continuous stirring, cooled to room temperature, and stored at 4 °C until use. The substrate solutions were dissolved in 50 mM Tris-HCl buffer (pH 7.0) to attain a specific concentration (3 mM). Before reaction, 0.1 mL of the substrate solution was added to each well of a 96-well microplate and the microplate was pre-incubated at 30 °C for 5 min in a microplate reader (SpectraMax iD3 Multi-Mode Microplate Reader, Molecular Devices Co., San Jose, CA, USA). The hydrolysis was initiated by adding 0.1 mL of the protein extract solution, and the absorbance at 410 nm ($\varepsilon$ = 18.3 $mM^{-1} \cdot cm^{-1}$) was monitored over the reaction time to quantify the amount of *p*-nitrophenol produced during the reaction. The standard curve of *p*-nitrophenol molar concentration versus the absorption unit (AU) was plotted using a *p*-nitrophenol standard (Supplementary Data S4). One unit of lipase activity was defined as the amount of lipase liberating 1 µmol of *p*-nitrophenol per minute.

### 3.4. Fluorometric Assay for Measuring Lipase Activity

The fluorometric assay for measuring the catalytic lipase activity was established using 4-MUO as an alternative substrate as previously described [34]. The substrate solution (50.00 mM) was prepared by dissolving 25 mg of 4-MUO into 1.1348 mL of dimethyl sulfoxide with vortexing. The solution was stored at −18 °C until use. The substrate solutions were dissolved in 50 mM Tris-HCl buffer (pH 7.0) to reach a specific concentration (3 mM). Before reaction, 0.1 mL of the substrate solution was added to each well of 96-well microplate and the microplate was pre-incubated at 30 °C for 5 min. The hydrolysis was then initiated by adding 0.1 mL of the protein extract solution. 4-methylumbelliferone fluorescence was detected at an excitation wavelength of 320 nm and an emission wavelength of 455 nm over the reaction time. The fluorescence data were acquired in bottom-reading mode and using photomultiplier tube (PMT) low measurement. The excitation and emission slit widths were set at 9 and 15 nm, respectively. The standard curve of 4-methylumbelliferone molar concentration versus the relative fluorescence unit (RFU) was plotted using 4-methylumbelliferone standard (Supplementary Data S5). One unit of lipase activity was defined as the amount of lipase liberating 1 µmol of 4-methylumbelliferone per minute.

### 3.5. Statistical Analysis

Statistical analyses were performed using SPSS statistics software (ver. 25.0; IBM Co., Armonk, NY, USA). The protein extraction yield data and specific activity ($n$ = 24) were subjected to one-way analysis of variance (ANOVA) according to the protein extraction methodologies, and the statistical significance between the values was validated using Duncan's multiple range test ($p < 0.05$).

## 4. Conclusions

In the present study, we comparatively investigated the efficiency of four universal protein extraction methodologies (ammonium sulfate precipitation, TCA/acetone precipitation, and two commercial kits) in terms of protein yield and specific activity to provide practical information regarding protein extraction to discover a novel lipase from agricultural products. This study indeed did not aim to develop or optimize protein extraction methodologies with a broad range of proteins or for experimental purposes because it

would be impossible due to the complexity and diversity of the proteins and their origins. Instead, we focused on providing several helpful, practical, and comprehensive results of protein extraction using commonly used methodologies in order to apply to high-throughput screening of enzymes, specifically that of lipases. Using 24 domestic agricultural products, TCA/acetone precipitation showed a relatively higher extraction yield in crude protein extraction, whereas the Pierce™ Plant Total Protein Extraction Kit showed the highest specific lipase activity in both the spectrophotometric and fluorometric assays. These results indicated that using commercial kits, especially the Pierce™ Plant Total Protein Extraction Kit, could be a more efficient strategy than conventional precipitation techniques to extract soluble functional proteins when performing high-throughput screening of enzyme activity in agricultural sources. Finally, several agricultural products such as cordyceps, pepper, bracken, and hemp, which exhibited an excellent specific activity of lipase in this study, would be promising candidates as a source of novel lipases.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/catal11070816/s1, Supplementary Data S1: Screening of lipase activity in 87 kinds of domestic agricultural products, Supplementary Data S2: Final candidates of domestic agricultural products (24 kinds), Supplementary Data S3: Standard curves of bovine serum albumin concentration versus absorption unit referring to the absorbance at 595 nm for Bradford assay, Supplementary Data S4: Standard curves of *p*-nitrophenol concentration versus absorption unit referring to the absorbance at 410 nm, Supplementary Data S5: Standard curves of 4-methylumbelliferone concentration versus relative fluorescence unit (RFU) referring to the emission fluorescence at 455 nm.

**Author Contributions:** K.-M.P. and P.-S.C. conceived the study and designed the experiments. J.H. performed the experiments and analyzed the data. J.-Y.P. and J.H. wrote the first draft of manuscript, and J.-Y.P., J.H. and Y.C. reviewed and revised the article. All authors discussed the contents of the manuscript and approved the submission. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The data used to support the findings of this study are available from the corresponding author upon request.

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