Use of Liquefied Dimethyl Ether for the Extraction of Proteins from Vegetable Tissues

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Dimethyl ether (DME), the simplest ether with the formula CH₃OCH₃, is a low-temperature solvent and extraction agent applicable to specialized laboratory procedures as recently demonstrated for extraction of biologically active, flavoring or pungent organic compounds from some biological materials. Due to its low boiling point, DME facilitates the removal of solvent from the samples after extraction. In the present study, we demonstrated the extraction of proteins from juicy or relatively dry vegetable tissues and the distribution of proteins in the dry phase and separated aqueous phase were compared. It is notable that a series of proteins from carrot roots, sized between 84.7 and 33.1 kDa, were detected in the liquid sample extracted by DME, suggesting that DME could be used as an effective extraction solvent for separating the hydrophilic (water soluble) proteins from the crude protein samples. Extraction of water-soluble proteins largely depends on the de-watering action of DME.

1. Introduction

Dimethyl ether (DME), the simplest ether with the formula CH₃OCH₃, is a colorless gas at ambient pressure. DME is a useful precursor to other organic compounds such as liquefied petroleum gas (LPG) [1] and small molecular hydrocarbons [2]. DME is known as a promising alternative fuel for diesel engines, petrol engines, and gas turbines [3]. DME appears to have the largest potential impact on society, and should be considered as the fuel of choice for eliminating the dependency on petroleum, compared to some
of the other leading alternative fuel candidates such as methane, methanol, ethanol, and Fischer-Tropsch fuels [4]. Notably, (i) DME can be used as a clean high-efficiency compression ignition fuel with reduced NOx, SOx, and particulate matter, (ii) it can be efficiently reformed to hydrogen at low temperatures, and (iii) DME does not have large issues with toxicity, production, infrastructure, and transportation as do various other fuels. Recently, the needs for and production of DME is rapidly increasing due to the above reasons [4].

Apart from its use as a fuel, subcritical DME has been shown to be an effective medium for extraction of biologically active, flavoring or pungent organic compounds from some spices (ginger, black pepper, and chili powder) and its effectiveness was shown to be comparable with that of supercritical carbon dioxide [5]. In addition to subcritical extraction protocol, DME can be used as a low-temperature solvent and extraction agent applicable to specialized laboratory procedures as recently demonstrated for extraction from several bio-materials [6]. Although the usefulness of DME as a solvent is limited by its low boiling point (-23 °C), this property could be beneficial in order to facilitate the removal of DME from the liquid mixtures and solids after extraction procedures.

Interestingly, Kanda and Makino [7] reported the use of liquefied DME in efficient de-watering procedures for sub-bituminous coal without any heating. The reported water extraction efficiency of liquefied DME was a maximum of 98.3%. This de-watering property and capacity in DME was also applied for dry matter preparation from highly-moist natural blue-green microalgae (91.0% of water content) [6].

In biochemical processes, acetone is used for the extraction of proteins from various biological samples and agricultural products. From the resultant “acetone powder,” enzymes can be readily extracted by suspending the powder in the extraction buffer [8]. In the present study, we compared the actions of acetone and DME as solvents for the extraction of proteins from freshly homogenized vegetable tissues. Lastly, distribution of proteins in a dry solid phase (powder) and a separated aqueous phase were compared.

2. Materials and Methods

2.1 Plant materials

Pericarp (fruits) of Japanese squash (Cucurbita moschata cv. Ebisu) and carrot roots (Daucus carota L., cv. Koyo-2) obtained from a local market was used for the sources of proteins. Squash pericarp were separated into peel (1 mm from the surface) and yellow tissue (green peel was used for extraction) while carrot roots were used without peeling. After homogenizing the tissues in the mixer, the obtained tissue paste (1-2 g fresh weight, gfw) was placed in the sample holder in the extraction chamber.

2.2 Apparatus for liquefaction of DME and protein extraction

The newly designed enzyme extraction apparatus consisted of (i) a DME gas cylinder connected to a
flow meter, (ii) a gas cooling unit, (iii) a column-shaped extraction chamber, and (iv) a liquid trapping unit (Figure 1 A). This system was originally used for extraction of pigments such as chlorophylls and carotenoids from fresh vegetables [9].

The column-shaped extraction chamber was composed of a 10 mL pressure-resistant glass column (inner diameter, 3.66 mm; height 950 mm) covered with a shielding column made of pressure-resistant polycarbonate resin (Hyper glass cylinder 10, Taiatsu Techno Corporation, Shizuoka, Japan; Figure 2B). The transparent extraction chamber allowed visual monitoring of loading and release of liquefied DME into and out of the extraction chamber, respectively. In the cooling unit, a combination of dry ice and acetone was employed as the cooling media which is known to achieve rapid cooling down to ca. -78 °C [10].

Figure 1. Apparatus for enzyme extraction with DME. (A) Composition of the system. (i) DME gas cylinder, (ii) cooling unit, (iii) Extraction chamber, and (iv) trapping unit are illustrated. Arrows indicate the positions of the two stopcocks inserted for flow control. (B, C) Extraction chamber. (D, E) Trapping unit.

Prior to the use of acetone, bottles of acetone were stored at -30°C for at least 1 day. The above units (i) - (iv) were connected to each other with aluminum capillaries. The DME gas cylinder was obtained from the local gas vendor. Within the extraction chamber, there is a disk-shaped sample holder on which samples could be set. The flow rate and duration of gaseous DME from the DME gas cylinder and supply
of liquefied DME from the cooling unit can be monitored with a flow meter (placed between (i) and (ii) in Fig. 1A) and visual inspection or monitoring of the filling of the extraction chamber (made of transparent acryl resin, Fig. 1B,C) with liquefied DME, respectively. Loading of the newly cryo-liquefied DME can be manually controlled by opening and closing the stopcocks placed above and/or below the extraction chamber (Figure 1A, two arrows).

From a preliminary examination employing a similar experimental set-up, the ratio of liquefied DME collected in the trapping tube over total liquefied DME loaded into the extraction chamber was determined to be ca. 57% (Hara et al., unpublished results), suggesting that the evaporation rate of DME inside the extraction apparatus was estimated to be ca. 43%. This suggests that the mixture of DME in liquid and gaseous forms likely passes through the vegetable samples. However, it is natural to assume that extraction can be solely attributed to the involvement of the liquefied portion of DME.

2.3 Treatment of plant materials with liquefied DME

For removal of water and lipid from the homogenized vegetable tissues, the apparatus shown in Fig. 1 was used. Gaseous DME supplied from the gas cylinder was rapidly cryo-liquefied in the cooling unit. Collection and release of liquid DME produced in the cooling chamber was controlled by opening and closing of a stopcock between the cooling unit and the extraction chamber. After loading ca. 15 ml of liquid DME into the extraction chamber, the sample was allowed to have contact with DME for a few seconds.

By opening the stopcock between the extraction chamber and the collection unit, DME and the accompanying liquid was collected in a test tube (Fig. 1D, E). Dry matter left in the extraction chamber was used as the source of proteins. The DME-dependently extracted liquid left in the trapping test tube which was collected immediately after extraction by DME was further subjected to passive DME removal under ambient pressure.

2.4 Preparation of acetone powder

Instead of treatment with DME, plant homogenates were used for preparation of acetone powder. Plant samples (pastes after homogenization) were suspended in 2 volumes of cold acetone and well mixed on an ice chilled mortar using an ice chilled pestle for ca. 5 min. The sample mixture was filtered through a Whatman filter paper placed on a Buchner funnel. The pellet were then repeatedly washed with cold acetone and dried overnight in vacuo.

2.5 Extraction of crude proteins

From the dry powder obtained after DME-based extraction or acetone powder preparation, soluble proteins were extracted by suspending the powder in the extraction buffer (25 mM potassium phosphate, pH 7.0). The resultant crude protein preparations were subjected to size analysis with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described below.

In addition to the proteins extracted from the dry matter, a portion of proteins DME-dependently removed from the dry matter during treatment with liquefied DME were also assessed. The DME-plant
extract mixture collected in the trapping unit was exposed to passive removal of DME under ambient temperature and the resultant liquid remaining in the test tube was shown to be a mixture of oil and water which were readily separated into distinct separate phases by centrifugation. Thus, the composition of proteins recovered in the hydrophilic (aqueous) phase was also assessed using SDS-PAGE.

### 2.6 SDS-PAGE

The composition of the proteins extracted by novel and conventional extraction procedures was evaluated by SDS-PAGE. Aliquots of the protein samples (from dry matter and extracted liquid) were analyzed by SDS-PAGE according to the method described by Laemmli [11]. The proteins were prepared in a loading buffer (0.5 M Tirs-HCl, pH 6.8, 10% SDS, 0.6% 2-mercaptoethanol, 1% glycerol and 1% bromophenol blue) without boiling. The proteins were separated on 10% acrylamide gel and exposed to staining with bromophenol blue, de-staining, and image analysis.

### 3. Results and Discussion

#### 3.1 Attempt to remove water and oil from the plant samples

Fresh tissues of Japanese squash (Figure 2 A, B; cortex with green peel) and carrot roots (Figure 2 F, G) were homogenized and the resultant pastes (Figure 2 C, H) were treated by extraction with liquid DME. Upon loading of liquid DME onto the layer of plant samples packed in the extraction chamber column, liquefied DME mixed for a few seconds with plant samples retained in the extraction chamber. After the de-watering process with liquefied DME, dry powder resembling the acetone powder commonly used for preparing the crude source of enzymes, was left in the apparatus (Figure 2 D, I). Then liquid that

![Figure 2. Fresh tissues, slices, homogenized paste, and dry powder of Japanese squash (cv. Ebisu; outer cortex with green peel) and carrot roots (cv. Koyo-2) used for DME-based protein extraction. Fresh tissues prior to homogenization (A, B, F, G), homogenates (C, H), and dry powder (D, I) used for protein extraction, and the liquid collected from the fresh samples as a consequence of the de-watering effect of DME (E, J) are shown.](image)
was mostly a mixture of extracted water, oil and liquid DME, was collected in the tubes placed beneath the extraction apparatus. As DME can be readily evaporated under ambient pressure and temperature, removal of DME from the collected liquid was passively allowed by leaving the tubes with liquid samples under ambient temperature for at least ca. 30 min. As a consequence, dense colored aqueous samples were shown to be left in the tubes (Figure 2 E, J), which are mixtures of oil and water indicating that both water and oils were removed from the plant samples by liquid DME.

Figure 3. Separation of soluble proteins by SDS-PAGE. Data from squash pericarp (peel) and carrot root are compared.

3.2 Analysis of protein extraction profiles by SDS-PAGE

By conventional protein extraction based on preparation of acetone powder, a series of proteins from the squash green peel, with bands of 111.3, 101.2, 91.7, 84.7, 81.5, 52.7, and 33.3 kDa, were obtained as major bands on SDS-PAGE although some tens of faint or minor bands could be visualized on the gel (Figure 3, left). In the protein extracts from the DME powder prepared from squash peel, only 4 major bands, namely, 84.7, 55.8, 55.0 and 33.3 kDa, were obtained, suggesting that DME powder is a relatively poorer source of protein compared to acetone powder. Among the bands of proteins extracted from the DME powder, bands at 84.7 and 33.3 kDa are commonly found in the acetone powder sample. Probably,
two additional bands at 55.8 and 55.0 kDa from DME powder samples could be also found as minor bands around the 52.7-kDa major band in the acetone powder sample, if assays with higher resolution were performed. In addition, no band of protein was detected in the liquid sample extracted by DME (Figure 3, left, third lane).

Similarly to the results in squash tissue, conventional protein extraction protocol by preparing acetone powder, allowed collection of a wide range of proteins from the carrot roots, compared to the DME powder sample from carrot roots (Figure 3, right). The lane for the acetone powder sample contained the bands of 94.8, 88.2, 80.1, 55.0, 54.1, 48.6, 46.7, 45.6, 44.5, 43.5, 42.5, 35.1, and 33.6 kDa proteins as major bands on SDS-PAGE while some additional of faint or minor bands could be visualized on the gel (Figure 3, right, first lane). In the protein extracts from the DME powder prepared from carrot roots, only 5 major bands, namely, of 94.8, 55.8, 54.1, 37.2 and 36.8 kDa, were obtained, again suggesting that DME powder is a relatively poorer source of protein compared to acetone powder. Among the bands of proteins extracted from the DME powder, bands of small proteins at 37.2 and 36.8 kDa could be possibly distinguished from the bands in the acetone powder sample (Figure 3, right, first and second lanes).

It is highly notable that a series of protein bands of sized 84.7, 73.4, 55.8, 54.1, 45.6, 45.0, 44.0, 35.1, and 33.1 kDa were detected in the liquid sample extracted by DME (Figure 3, right, third lane). Interestingly, the bands at 84.7, 73.4 could be hardly recognized in the gel of either the acetone powder sample or the DME powder sample. Since these proteins were found in the hydrophilic fraction in the liquid sample extracted by DME, they are all water soluble proteins, suggesting that DME could be used as an effective extraction solvent for separating the hydrophilic (water soluble) proteins from the crude protein samples. However, this protocol should be applicable only to watery samples like juicy berries, fruits and vegetables since the likely mechanism for extraction of water soluble proteins largely depends on the de-watering action of DME as certain portions of protein accompanies the movement of water.

### 3.3 Conventional and unconventional protein extraction protocols

The acetone-mediated enzyme extraction is based on the precipitation and binding of proteins on the surface of dry matter in acetone. Then, collection of hydrophilic (water soluble) and hydrophobic (water insoluble) proteins from the acetone powder requires further extraction of protein from the powder by addition of buffers with and without detergents, respectively [12]. As demonstrated here, two distinct modes of DME-mediated enzyme (protein) extractions can be developed, namely, one based on the extraction of dry matter-bound proteins similar to the manner performed with acetone, and one based on the isolation of a water soluble protein fraction through the de-watering action of DME. However, further studies on the nature of proteins in the DME-extracted fraction recovered during the de-watering action of DME, are highly encouraged since there is a possibility that the DME/water mixture helps the extraction of amphiphilic proteinaceous molecules or complexes soluble in both DME and water. Comparison with the protocols employing surfactants is one of the approaches to determine the nature of DME-extracted and
DME powder-bound proteins.

Since surfactants including those derived from fatty acid salts are often used for enhancing the solubility of proteins or conversely for promoting the gelation of proteins in aqueous media, some specific detergents are applicable for rapid separation of proteins from biological samples. We have previously developed an unconventional protocol for rapid separation of proteins from aqueous media using a novel detergent-gelation protocol, in which protein in aqueous media (with hydrophilic and hydrophobic natures) can be readily removed by addition of a soap (fatty acid salt)-based detergent cocktail and specific salts [13]. In the demonstrations performed, human hemoglobin, serum proteins, and mitochondrial proteins were successfully collected on solid discs floating over the solution following simple centrifugation.

The present report also presents a novel unconventional protocol for extraction of proteins from the plant tissues. Since the experiment can be accomplished under cooling conditions, one might expect that the biological activities of the obtained proteins such as enzymes can be preserved. Therefore, further studies are required to confirm this view. At the moment, the authors have obtained some preliminary data showing that the activity of chlorophyllase an enzyme responsible for breakdown of chlorophylls from the citrus peels, is present in significant amounts in the DME-powder preparation compared to acetone powder preparation. In addition, the soluble fraction directly recovered by DME also contains active enzymes (data not shown).

Therefore, use of liquefied DME in extraction of proteins from a variety of biological samples are highly encouraged.

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References