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Preliminary Evaluation of an Ultrafiltration System to Minimize Phenolic Interference during Protein Extraction and Fractionation

L. S. Bates[a]
[a] DEPARTMENT OF GRAIN SCIENCE AND INDUSTRY, KANSAS STATE UNIVERSITY, MANHATTAN, KANSAS

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NOTE

Preliminary Evaluation of an Ultrafiltration System to Minimize Phenolic Interference during Protein Extraction and Fractionation

L. S. BATES

DEPARTMENT OF GRAIN SCIENCE AND INDUSTRY
KANSAS STATE UNIVERSITY
MANHATTAN, KANSAS 66506

Abstract

Preliminary evaluation of an ultrafiltration system for protein/phenolic mixtures demonstrated interactions were greatly limited by extraction and separation of larger MW proteins from phenolics in aqueous 5% DMSO. A 10,000 MW cut-off filter was the most useful to separate proteins from phenolics, and no filter sustained visible damage from 5% DMSO. A cellophane dialysis bag might be equally useful. Some proteins reacted with phenolics in the 1000 to 10,000 MW fraction after dimethylsulfoxide (DMSO) removal, which demonstrates DMSO protection of proteins in the presence of phenolics. The ampholyte shift can serve as a relative subjective measure of free phenolic presence. The total system requires additional refinement but demonstrates DMSO protects protein at a concentration lower than previously reported (5% vs 10 to 90%), and it may be effective as low as 1% or less.

INTRODUCTION

One of the most difficult and underestimated problems in natural product chemistry is the separation of proteins from phenolics. Phenolics are ubiquitous in nature and react rapidly with proteins upon disruption of the strict compartmentalization of cells. Although protein/phenolic reactions occur commonly, their extent and nature are not well understood (*1*). Small molecular weight phenolics may be absorbed onto proteins but because their complexes are not particularly stable, they

cause minimal difficulties. Larger molecular weight phenolics, primarily tannins (500 to 3000 MW) containing one to two phenolic hydroxyl groups per 100 MW (*2*), create the major problems via stable cross-links with proteins. These resulting complexes may form precipitates or colloidal suspensions of denatured protein or they may merely produce temporarily inactivated proteins/enzymes.

Phenolic compounds react generally in one of two ways under "physiological" extraction conditions. Hydrogen bonding, in which all nonsterically hindered phenolic hydroxyl groups participate, is the more common of the macromolecular interactions. These strong hydrogen bonds, formed primarily via peptide oxygen molecules, are reversible at approximately pH 8 when the phenolic hydroxyl is ionized. A second general reaction is the irreversible condensation of oxidized phenols (quinones) with sulfhydryl, free amino acid, or free amino groups of proteins via covalent bonds. Quinones, effective oxidizing agents, may oxidize other essential functional groups of proteins also (*3*).

Absolute protection against phenolic/protein reactions and interactions during extractions is virtually impossible. Hydrogen bonding difficulties may be resolved by adjusting pH for phenolic hydroxyl ionization; by using selective insoluble substitute binding agents such as polyvinylpyrrolidone (PVP), synthetic resins, or hide powder; or by using strong hydrogen bond acceptors in the extracting solvents (*3*, *4*). Quinone formation, which can be caused by numerous enzymes and nonenzymatic agents, cannot be prevented although reducing agents may limit polymeric condensation and oxidative side reactions. Because reducing agents may be deleterious to some proteins, quinone protection must be evaluated against the adverse effects of reducing agents.

This report concerns the development, application, and preliminary evaluation of an alternative hydrogen bond acceptor approach following the suggested use of dimethylsulfoxide (*3*) combined with gradient elution through a tandem configuration of stirred ultrafiltration cells and reservoirs.

MATERIALS AND METHODS

Maize (*Zea mays* L.) pollen was collected, screened of foreign debris, and refrigerated at 5 °C for no more than 4 days before extraction. Samples (10 mg pollen) were extracted with 10 ml of either distilled water or 10% aqueous dimethylsulfoxide (DMSO) in a glass hand homogenizer cooled

in an ice bath. The extracts were diluted with an equal volume of 10% DMSO or distilled water, respectively, to produce uniform 5% DMSO solutions and stored at 5°C. Other 10 mg samples were extracted with 10 ml of 5% DMSO, filtered through a glass fiber filter pad, centrifuged at 12,750 rpm (20,000 rcf) in a Beckman J-21 centrifuge, and fractionated via individual ultrafiltration (UF) cell steps.

Subsequent modification of the individual UF cell system involved a DMSO/water gradient extraction of pollen in UF cells coupled as a tandem cascade similar to Blatt (*5*). Extraction and fractionation were completed simultaneously with the following Amicon Corp. membranes in descending order: microporous 0.2 μm, XM-100A, PM-10, and UM-2. The whole system was sterilized and assembled in a laminar flow chamber with 40 ml distilled water in each cell. The cascade was preceded by a 0.2-μm equipped stirred cell containing 10 mg pollen in 10 ml 5% DMSO and gradient eluted with distilled water to <0.05% DMSO (Fig. 1).

All extracts and fractions were stored at −10°C until electrofocused. The cathodic ampholyte shift was used to gauge the relative amount of protein/phenolic reaction.

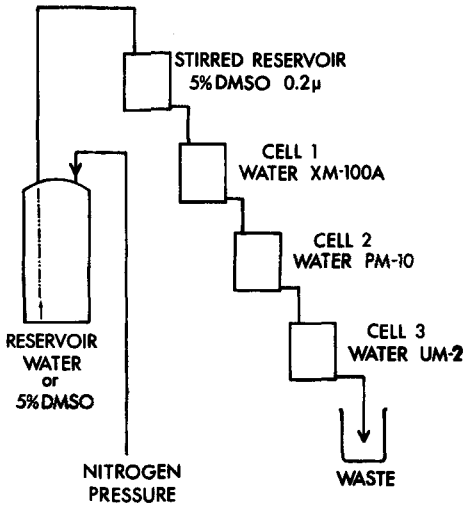

FIG. 1. Diagram of tandem cascade for continuous extraction, diafiltration, and fractionation of maize pollen proteins. The pollen sample is located in the stirred reservoir.

RESULTS AND DISCUSSION

Maize pollen was chosen as an easily defined, collected, and manipulated material containing relatively large amounts of quercitin and other polyphenolic compounds along with 20 to 25% protein (*6–8*). Upon lysis and extraction, an ideal protein/phenolic reaction milieu should result.

Because DMSO had been suggested as an alternative hydrogen bond acceptor (*3*) and Ascher and Weinheimer (*9*) had demonstrated the stability of crude protein extracts from numerous sources in 10 to 90% DMSO, aqueous dimethylsulfoxide was selected for these studies. DMSO had also been reported to inhibit reversibly the enzymes for pollen tube growth (*10*), to inhibit metabolism primarily via respiration (*11*), and to stabilize lipoproteins in low-temperature experiments (*12*). Additionally, DMSO is an excellent solvent of many materials (*13*) and is not recommended for use with UF membranes.

The first consideration was to extend reported protein stability observations (*9*) to lower DMSO levels. Protein stability in 5% aqueous DMSO was examined following either 10% DMSO or water extraction. Within 24 hr a proteinaceous precipitate began to settle out of the water/5% DMSO extracts whereas the 10% DMSO/5% DMSO extracts remained clear for 2 weeks before any precipitation occurred. Dilutions, 1 to 5 and 1 to 50, of the latter extract with water caused no additional precipitation. Similar dilutions of the water/5% DMSO extract did not dissolve the precipitate. The results suggest protein/phenolic reactions, that were initiated immediately on cell disruption, were not reversible by DMSO addition. Proteins were protected from irreversible reactions, presumably with phenolics, only when lysed, homogenized, and stored in aqueous DMSO. Direct 5% DMSO extractions remained clear and stable also.

A second consideration was UF membrane stability and the separation of proteins from phenolics while protected in aqueous DMSO. Because reduced water flow rates occur after 24 hr of 5% DMSO soaking, MW cut-offs may change during fractionation (Amicon Technical Services, Personal Communication). Consequently UF pressures were kept low and no critical conclusions were based on MW. Extracts were diafiltered at 5°C with 5% DMSO at 25 to 30 psi. Membrane polarization occurred with sample concentration but no UF membrane damage was observed.

Evaluation of protein/phenolic interactions was based on the ampholyte shift of polyacrylamide gel isoelectrofocusing (PAGIF). Dimethylsulfoxide effectively substitutes for *N*, *N*, *N'*, *N'*-tetramethylenediamine (TMED), ampholytes, and sample in riboflavin/light-catalyzed acrylamide polym-

erization and does not affect the ampholyte shift. Conversely, phenolics tends to inhibit free radical polymerization and cause soft gels, electroosmosis, and ampholyte shifting (*14*). Thus a relatively simple subjective system to visualize the presence of free phenolics was based upon acrylamide polymerization and ampholyte shifting. The two largest MW fractions (<100,000 MW and 10,000 to 100,000 MW) could be polymerized readily whereas the 1000 to 10,000 MW fraction, containing most of the tannins and other phenolics, gelled with great difficulty or remained liquid. Subsequent PAGIF results suggested the interferring phenolics were effectively separated from the majority of the larger MW pollen proteins by negligible ampholyte shifting.

The final consideration was to diafilter the DMSO with water to determine if suspected free phenolics would react with protein in the same fraction. The gradient varied in practice because of the tandem cascade, but it was designed to approximate Curve B of O'Sullivan's constant volume elution method with 2 stirred reservoirs (*15*). An accurate gradient was not necessary as it would not influence extraction or fractionation. It served only as part of the wash-out for DMSO while extending protein protection against phenolics. Unfortunately, the 1000 to 10,000 MW fraction gelled, indicating the free phenolics reacted with the protein when the DMSO level was reduced to <0.05%. Results of the larger MW fractions were as before. A single dialysis in cellophane against 1 to 5% DMSO or diafiltration with a PM-10 membrane should be equally effective although the latter would be faster.

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