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# EXTRACTION OF LEAF PROTEINS OF *MENTHA* (LABIATAE) FOR DISC-ELECTROPHORESIS

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**Abstract**—"Browning" of leaf extracts from *Mentha* species prevents accurate analysis of the proteins by disc-electrophoresis. Seventeen reagents were compared to determine minimum browning and maximum extraction of proteins; pre-extraction of phenols with aqueous acetones was also investigated. 2-Mercaptoethanol, sodium mercaptoacetate, sodium ascorbate, L(+)-cysteine, sodium hydrosulfite, dithioerythritol, sodium *N,N*-diethyldithiocarbamate, and potassium metabisulfite were demonstrated to be effective for extraction of protein, but all these reagents except 2-mercaptoethanol interfered with disc-electrophoresis. Pre-extraction of phenols from fresh leaves with 80 per cent acetone prepared with an alkaline buffer or distilled water was also demonstrated to be effective for extraction of protein, but the disc-electrophoretic bands were less distinct than those obtained with 2-mercaptoethanol.

## INTRODUCTION

THE PROBLEMS encountered in extracting proteins from vegetative portions of plants have been adequately reviewed.[1,2] The basic problem involves "browning", or oxidation of phenols to quinones and subsequent precipitation of proteins with quinones by covalent bonding.[1]

This difficulty in extracting proteins from leaves of *Mentha* was apparent when fresh leaves browned on being ground in a mortar and pestle; and variable, irreproducible results were obtained with disc-electrophoresis.[3] Pshenova,[4] Loomis and Battaile,[1] and Crawford[5] have reported active phenol oxidases in leaves of *Mentha*, while the phenols have been characterized by Hörhammer and Wagner[6] and Gella *et al.*[7-9]

Since the phenol oxidases could not be pre-isolated in pure form without further information on the enzymes or substrates present in *Mentha*, the following seventeen phenol-removing, phenol-oxidase-inhibiting, and quinone-removing reagents[2] were experimentally compared in serial dilutions from 1/1 to 1/1024 to determine the best method of protein extraction: 1. 1·0 M 2-mercaptoethanol (2-EtSH), 2. 1·0 M sodium mercaptoacetate (sodium thioglycolate), 3. 1·0 M reduced glutathione (GSH), 4. 1·0 M 2-mercaptobenzothiazole

[1] W. D. LOOMIS and J. BATTAILE, *Phytochem.* **5**, 423 (1969).
[2] J. W. ANDERSON, *Phytochem.* **7**, 1973 (1968).
[3] D. E. FAIRBROTHERS, *Rev. Roumaine Biochim.* **6**, 95 (1969).
[4] K. V. PSHENOVA, *Biochem.* **21**, 281 (1956).
[5] R. M. M. CRAWFORD, *Nature* **214**, 427 (1967).
[6] L. HÖRHAMMER and H. WAGNER, in *Recent Progress in the Chemistry of Natural and Synthetic Colouring Matters and Related Fields* (edited by T. S. GORE, B. S. JOSHI, S. V. SUNTHANKAR and B. D. TILAK), p. 315, Academic Press, New York (1962).
[7] E. V. GELLA, Y. H. BORISYNK and G. V. MAKAROVA, *Farmatsevt Zh.* **20**, 31 (1965).
[8] E. V. GELLA, G. V. MAKAROVA, Y. H. BORISYNK and V. I. LYTVYNENKO, *Farmatsevt Zh.* **21**, 58 (1966).
[9] E. V. GELLA, G. V. MAKAROVA and Y. H. BORISYNK, *Farmatsevt Zh.* **22**, 80 (1967).

(MBT) (only 1/1024), 5. 1·0 M sodium ascorbate, 6. 1·0 M L(+)-cysteine-HCl monohydrate, 7. 1·0 M dithioerythritol (DTE), 9. 1·0 M tetrasodium ethylenediamine tetra-acetate (Versene), 10. 1·0 M sodium *N,N*-diethyldithiocarbamate (DIECA), 11. 1·0 M potassium metabisulfite, 12. 1·0 M caffeine (only 1/16–1/1024), 13. 1·0 M nicotine, 14. 10 per cent (v/v) Tween 80, 15. 10 per cent (w/v) polyvinylpyrrolidone (PVP), 16. 10 per cent (w/v) Polyclar AT (a partially insoluble polymer of PVP) (only 1/2–1/1024), and 17. 10 per cent (w/v) Norit-A (a purified charcoal). Experiments were also conducted to test the effect of pre-extraction of phenols with 80 per cent acetone prepared with distilled water or an acid or alkaline buffer.

The experiments were designed to obtain data which could be evaluated to determine the minimum concentration of reagent for 1. maximum amount of proteins extracted; 2. maximum number of proteins extracted; 3. maximum prolongation of phenol-removing, phenol-oxidase-inhibiting, or quinone-removing; 4. minimum enzyme denaturation; and 5. minimum interference with disc-electrophoresis. For quantification, the method of Lowry *et al.*[10] was chosen over that of Kjeldahl analysis of total nitrogen because PVP is nitrogenous and is precipitated in any attempt at purification. Since phenols give a positive reaction with the Lowry method and are precipitated with acid or salt,[11] purification with acetone, similar to the method employed by Lyttleton[12] and Potty[11] for ethanol, was used. However, any quantification alone would mostly measure the amount of Fraction I protein, or ribulose-1,5-diphosphate carboxylase, extracted, which may commonly comprise up to 50 per cent[13] or even 75–80 per cent[14] of the proteins in an aqueous extract.

The number of proteins extracted was measured directly by disc-electrophoresis, thereby screening those reagents which interfere with electrophoretic mobility.[15,16] Denaturation was measured by the activity of alkaline phosphatase[1] of the serial dilution of the reagent indicated best by the above procedures.

Reagents were diluted in 0·05 M sodium borate buffered 0·2 per cent saline pH 8·0. Even though removed from the p$K_a$'s of boric acid, a borate buffer is qualified by the data of Goldstein and Swain[17] and Adatthody and Racusen.[18] To reduce the variance of different physiological states between harvests and minor alterations in purification, a control of 0·25 M 2-mercaptoethanol was included in each extraction. No replicates of the dilutions were performed.

## RESULTS

Prevention of browning and maximum extraction of proteins from acetone leaf powders were definitely enhanced by the inclusion of 2-mercaptoethanol, sodium mercaptoacetate, sodium ascorbate L(+)-cysteine, sodium hydrosulfite, dithioerythritol, sodium *N,N*-diethyldithiocarbamate, or potassium metabisulfite in 0·05 M sodium borate buffered 0·2 per cent saline, pH 8·0. However, all of these reagents except 2-mercaptoethanol interfered with disc-electrophoresis. The optimum results, as indicated by a serial dilution of 1·0 M 2-mercaptoethanol analyzed by disc-electrophoresis, total protein, and alkaline phosphatase activity were produced by 0·25 M 2-mercaptoethanol when acetone leaf powders were extracted in the ratio of 0·1 g:1 ml.

[10] O. H. LOWRY, N. J. ROSEBOROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[11] V. H. POTTY, *Anal. Biochem.* **29**, 535 (1969).
[12] J. W. LYTTLETON, *Biochem. J.* **64**, 70 (1956).
[13] L. EGGMAN, S. J. SINGER and S. G. WILDMAN, *J. Biol. Chem.* **205**, 969 (1953).
[14] S. G. WILDMAN and J. BONNER, *Arch. Biochem.* **14**, 381 (1947).
[15] J. M. BREWER, *Science* **156**, 256 (1967).
[16] B. LERCH and H. STEGEMANN, *Anal, Biochem.* **29**, 76 (1969).
[17] J. GOLDSTEIN and T. SWAIN, *Phytochem.* **4**, 185 (1965).
[18] K. K. ADATTHODY and D. RACUSEN, *Can. J. Botany* **45**, 2237 (1967).

Pre-extraction of phenols from fresh leaves of *Mentha* with 80 per cent acetone prepared with a slightly alkaline buffer or distilled water offered another means of maximum protein extraction and minimum browning, but the disc-electrophoretic bands were not as distinct as those obtained with 0·25 M 2-mercaptoethanol.

## DISCUSSION

Increasing emphasis is being placed on the employment of plant proteins for systematic studies.[3] Vegetative proteins, in particular, offer several advantages: 1. their availability throughout most of the plant's life cycle; 2. their availability in cases of sterility or of low seed or pollen production; 3. their assurance of uniform genotype by clonal propagation; and 4. their comparability in certain cases, especially ribulose-1,5-diphosphate carboxylase, throughout many photosynthetic plants.[19] However, certain precautions are necessary before any infraspecific or interspecific studies are attempted, as the quality and quantity of vegetative proteins may vary according to ontogenetic development,[20,21] season,[22] photoperiod,[23] or wilting.[24] An additional variation was indicated by our preliminary studies: that associated with extraction procedures. In view of the wide distribution of phenol oxidases,[25] the prevention of browning for the extraction of vegetative proteins is not limited to *Mentha*. Previous studies, in many cases, have ignored either the prevention of browning or any attempt at physiological comparability.

Our recommendation of the addition of 0·25 M 2-mercaptoethanol is not to be interpreted as universally applicable for analysis of vegetative proteins by disc-electrophoresis. Rather, any method of extraction must be critically evaluated in terms of the species and reagents involved.

## EXPERIMENTAL

### *Plants and Culture*

*Mentha* "61–1194", a synthesized hybrid of *M. rotundifolia* Huds. ($4n = 48$) and *M. longifolia* Huds. ($4n = 48$), was employed in all extractions. Ramets of this hybrid were grown in plastic pots containing washed quartz sand and flushed on alternate days with the complete nutrient solution of Hoagland and Arnon[26] dissolved in tap water. The normal day-length of an intensity of 33,000–55,000 lx in the greenhouse was extended to 13·5 hr of an intensity of 1100–2800 lx with incandescent lights. Temp. and humidity during the day ranged from 18–38° and 54–84% and during the night from 16–28° and 64–84%.

### *Harvesting and Preparation*

Mature (fully expanded) leaves were harvested at 3.00–4.00 p.m., immediately rinsed in distilled water, blotted dry, and then processed by a modified method of Morton.[27] In this technique the leaves were ground with liquid $N_2$ in the Prolabo Microbroyeur Quantitatif Damgoumau for 2 min and immediately immersed for 1 hr in acetone chilled to −27° in the ratio of 100 g of fresh leaves/500 ml. They were then washed on Whatman No. 1 filter paper with chilled acetone until a clear filtrate was obtained, immersed for 20 min in 200 ml of *n*-butanol chilled to −27°, and then centrifuged for 20 min at 4580 *g* and −20°. After rinsing with chilled acetone, the powder was dried for 12 hr at −27° in a freezer with a frost-removal system. All powders were sealed in glass vials and stored under vacuum over $P_2O_5$ at −27°.

[19] R. W. Dorner, A. Kahn and S. G. Wildman, *Biochim. Biophys. Acta* **29**, 240 (1958).
[20] R. W. Dorner, A. Kahn and S. G. Wildman, *J. Biol. Chem.* **229**, 945 (1957).
[21] N. Kawashima, A. Imai and E. Tamaki, *Plant Cell Physiol.* **8**, 447 (1967).
[22] L. C. van Loon and A. van Kammen, *Phytochem.* **7**, 1727 (1968).
[23] R. M. Warner and M. D. Upadhyda, *Physiol. Plantarum* **21**, 941 (1968).
[24] D. Frohne, *Bull. Serol. Mus.* **40**, 7 (1968).
[25] M. W. Onslow, *Biochem. J.* **15**, 107 (1921).
[26] D. R. Hoagland and D. I. Arnon, *Calif. Agr. Exp. Sta. Circ.* 347 (1950).
[27] R. K. Morton, in *Methods in Enzymology* (edited by S. P. Colowick and N. O. Kaplan), Vol. 1, p. 25, Academic Press, New York (1955).

The above procedure was used unaltered for comparison of the seventeen reagents. For pre-extraction of phenols, however, the procedure indicated by Table 1 was applied to both the fresh leaves and acetone powders.

TABLE 1. FLOW SHEET FOR PRE-EXTRACTION OF PHENOLS FROM FRESH LEAVES AND ACETONE POWDERS

| Treatments | Samples and reagents | | | | | |
|---|---|---|---|---|---|---|
| I | 1,2 | | 3,4 | | 5,6 | |
| | + | | − | | O | |
| II | 1 | 2 | 3 | 4 | 5 | 6 |
| | − | O | + | O | + | − |
| III | O | O | O | O | O | O |
| IV | A | A | A | A | A | A |
| V | N | N | N | N | N | N |
| VI | A | A | A | A | A | A |

Reagents

+ = 80% acetone prepared with 0·1 M sodium citrate, pH 4·0, at 26° and chilled to −27°
− = 80% acetone prepared with 0·05 M sodium borate buffered 0·2% saline, pH 8·0, at 26° and chilled to −27°
O = 80% acetone prepared with deionized, distilled water and chilled to −27°
A = acetone chilled to −27°
N = *n*-butanol chilled to −27°

Treatments

I = 100 g fresh leaves/500 ml at −27° for 60 min and 1000 ml over Whatman No. 1 or 1 g acetone powder/25 ml at −27° for 60 min and 50 ml over Whatman No. 1
II = 250 ml (fresh leaves) or 12·5 ml (acetone powder) over Whatman No. 1
III = 250 ml (fresh leaves) or 12·5 ml (acetone powder) over Whatman No. 1
IV = 500 ml (fresh leaves) or 25 ml (acetone powder) over Whatman No. 1
V = (fresh leaves only) 25 ml for 20 min, and then centrifuged for 20 min at 4270 *g* and −20°
VI = (fresh leaves only) 250 ml over Whatman No. 1

*Extraction*

All 1/1 concentrations of reagents were adjusted to pH 7·5–8·0 with HCl or NaOH prior to serial dilution in 0·05 M sodium borate buffered 0·2% saline, pH 8·0, with 0·01% Merthiolate, except for those with PVP or Polyclar AT, which were adjusted to pH 7·0. Acetone powders prepared with 80% acetone were extracted in plain buffer. All powders were extracted for 1 hr at 2° in the ratio of 0·1 g:1 ml, centrifuged for 20 min at 11,000 *g* and 2°, and the supernatant Millipore filtered (1·2 $\mu$).

*Disc-electrophoresis*

Disc-electrophoresis was performed according to Ornstein[28] and Davis[29] on the Buchler Poly-Analyt with 7% alkaline polyacrylamide gels and buffers prepared according to Canalco.[30] Twelve sample tubes were run at 36 mA/twelve tubes until the lead band of bromthymol blue migrated to within 1 cm of the bottom. Gels were stained for at least 12 hr in 0·2% aniline blue-black in 7% acetic acid and destained electrophoretically at 96 mA/twelve tubes in 7% acetic acid. Band measurements were immediately recorded before the gels were removed from the destaining tubes, from the interface of the stacking and separating gels to the leading edge of each band. $R_p$ values[31] were determined in relation to the lead band.

[28] L. ORNSTEIN, *Ann. N.Y. Acad. Sci.* **121**, 321 (1964).
[29] B. J. DAVIS, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
[30] CANALCO CO., *Chemical Formulation for Disc Electrophoresis*, Bethesda, Maryland (1965).
[31] J. G. VAUGHAN, A. WAITE, D. BOULTER and S. WAITERS, *J. Exptl. Botan.* **17**, 332 (1966).

*Protein Quantification*

The extract was cooled to 0°, precipitated with 67% acetone chilled to −27°, and centrifuged for 20 min at 4270 *g* and −20°. The precipitate was washed with twice the original volume of 80% acetone at −27° and centrifuged for 20 min at 4270 *g* and −20°. Samples with sodium ascorbate, sodium hydrosulfite, and L(+)-cysteine received an additional washing with twice the original volume of 20% trichloracetic acid at 2° and were centrifuged for 20 min at 4270 *g* and 2°; samples with reduced glutathione received an additional washing with 85% saturated ammonium sulfate at 2°. All samples were redissolved in 0·1 N NaOH at 26°. Quantification was performed according to Lowry *et al.*[10] employing bovine serum albumin as the standard.

*Alkaline Phosphatase Activity*

Extracts were purified on Sephadex G-50 "fine"[22] equilibrated with 3 mM Tris-acetate, pH 8·0. Alkaline phosphatase activity was analyzed according to Loomis and Battaile.[1]

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