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PRINCIPAL MODIFICATIONS IN THE DISC ELECTROPHORESIS OF PROTEINS AND ISOENZYMES OF GOLDEN DELICIOUS AND COX'S ORANGE PIPPIN APPLES

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1. Introduction

The objectives of this rapport are:

1. To mention the principal modifications of the polyacrylamide gel electrophoresis (Maurer, 1971) carried out for isoenzymes in apples. This will facilitate the work when repeated in the future.
2. By making the modifications accessible to the reader, we can publish in an International Journal the results on apples without giving all the details of the method.

2. Principal abbrevations

Tris	Tris(hydroxmethyl)aminiomethane as base
TEMED	N,N,N',N'-tetramethylethylenediamine
Bis	N,N'-methylene-bis-acrylamide
DAB	3,3'-Diaminobenzidine
H_2O	distilled water

3. Experimental

The complete installation of Shandon, England (MKII) for disc electrophoresis was used.

3.1 Preparation of gels

3.1.1 Separating gel (see annex 2)

The soln. was prepared in a small Erlenmeyer (ca. 50 ml) in presence of 1-2 glass pearls of 3mm diameter. The air was removed with a water vacuum pump and directly poured into the tubes until 15mm lower than the superior borders of the tubes. They were closed in the inferior orifice with parafilm and introduced in a rubber holder. Precautions were taken that the length of the polymerized gel was always the same. Afterwards with an enlarged pasteur pipette H_2O was added (2-3 mm above the gel) in order to avoid concavity of the superior surface of the gel. Water was so carefully poured that it did not mix with the gel. Furthermore the tubes were in perfect vertical position. Then chemical polymerization occurred. After 20-30 mins. the H_2O (2-3mm) was removed and its place, was rinsed twice with spacer gel soln (annex 2), freshly prepared.

3.1.2 Spacer gel (see annex 2)

On the said tubes containing the separating gel, spacer gel soln. was poured. Afterwards water was added to avoid concavity of

the spacer gel. When adding water the following was done in order to elude mixing of the water with the gel: water was added with an enlarged pasteur pipette being gradually displaced along the interior side of the tube with an ascending movement. In other words the pipette was continuously against the wall of the tube.

It was necessary that the period of time between the preparation of spacer gel and start of electrophoresis was not higher than 3 hours.

After again controlling for verticality of the tubes, the photopolymerization (lamp Chromatolux 254 nm, Bleuger) was started immediately for 20 min at a distance of 4-5 cm from the lamp. The layer of water accumulated in the superior part of the tube was removed, i.e. after ending of photopolymerization.

3.2 Electrophoresis

After polymerization the parafilms were removed from the inferior orifice of the tube and directly introduced into the upper buffer reservoir. Then the samples of the apple (solutions obtained from the acetone powder) were poured on the spacer gel. The upper and lower buffer reservoirs were filled with electrode buffer soln. pH 8.3 (see annex 3), diluted 10x previous to be immersed the electrode. It was important to observe that in the superior, (as well as in the inferior) part of the tubes there was no air. Air would be present when there was concavity of the gels. Moreover in the upper reservoir it was pipetted ca. 1 ml of bromphenol blue soln. (annex 1). The electrophoresis was carried out in the cold room (4°C), 2 - 2.5 mA per tube, until the bromphenol blue reached a distance of 2 mm from the bottom. In the said conditions the electrophoresis lasted approx. 2 hours. Afterwards the gels were removed from the tubes with a needle connected to a syringe. At least triplicate experiments were always performed at the standardized conditions mentioned.

3.3 Staining methods for proteins and isoenzymes

3.3.1 Proteins

The gels were removed from the tubes and introduced into a fixing solution (annex 3). After one hour they were immersed in a Soln of Coomassie Brilliant Blue R250 (annex 3) at 60°C for 10 min. Then the gels were destained in 5% acetic acid for 2-3 days. The gels with clear bands of proteins were kept for a long time (6 months) in 5% acetic acid.

3.3.2 Peroxidase isoenzymes

The staining method of Herzog and Fahimi (1973) slightly modified was applied. The amount of H_2O_2 soln. was 3.5 ml instead of 1 ml (see annex 3) and in addition gelatine was not used.

The staining process lasted ca. one hour at room temperature and in dark. Afterwards the gels were removed from the staining soln and put in water. When poured into water the gels enlarged from ca. 6.5 cm to 7.5 - 8 cm.

3.3.3 Esterase isoenzymes

The method of Rudolph and Stahmann (1966) was preferred above the one mentioned by Maurer (loc.cit.).

The gels were incubated in the staining soln of esterase (annex 3) for 20 min at room temperature and in dark to avoid precipitation of the Fast Blue RR. (The said soln of annex 3 was filtered through filter-paper Carl Schleicher and Schull (Germany) no. 595, before adding the 1% α-naphtyl acetate soln in acetone).

After incubation the gels were removed and kept in a preserving soln of H_2O: methanol: acetic acid (5:5:1 v/v). The isoenzymes were grey colored.

The R_m was calculated immediately because the preserving soln contracted (shrinked up) the gels.

A solution without substrate (α-naphtylacetate) was used as appropriate blank.

3.3.4 Amylase isoenzymes

It was done according to the method of Macko, Honold and Stahmann (1967) but the concentration of the starch in the gel was of 0.075% instead of 0.3%.

After electrophoresis the gels were incubated overnight in 0.2 M acetate buffer pH 4.8 at room temperature; and stained at 25°C with staining soln of annex 3. After 10 min the gels were kept in H_2O. The respective blank was without iodine.

The bands with amylase activity did not stain (negative coloration). The picture was stable for 2-3 hours. Therefore photos or documentation was done within the said period of time.

3.3.5 Catalase isoenzymes (see annex 3, staining soln for catalase)

The method of Macko, Honold and Stahmann (loc.cit.) was applied with the following modifications:

1. After electrophoresis the gels were incubated in soln a for 2-3 hours at 30°C and rinsed twice with H_2O.
2. Afterwards the gels were submerged in the solns. b and c that were previously mixed 1:1 (v/v).

After 4-5 mins the bands with catalase activity were observed (negative staining). The gels were kept in H_2O. Appropriate blank was without H_2O_2.

The bands were stable for 2-3 hours. Consequently photos or documentation were made directly.

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Annex 1 Stock solutions. They were kept at 2-4°C in brown bottles.

(A) pH 8.9

HCl, 1 N		48 ml
TRIS	(Merck, Art. 8382)	36.6 g
TEMED	(Serva, Art. 35925)	0.46 ml
H_2O to final volume of		100 ml

(B) pH 6.9

1 N H_3PO_4	25.6 ml
TRIS	5.7 g
H_2O to a final volume of	100 ml

(C) 12% (kept maximum for 20 days)

Acrylamide	(Serva, Art. 10674)	48 g
Bis-acrylamide	(Serva, Art. 29195)	0.8 g
H_2O to a final volume of		100 ml

(C_1) 12% (kept maximum for 20 days)

Acrylamide		48 g
Bis-acrylamide		0,8 g
$K_3Fe(CN)_6$	(Merck, Art. 4973)	15 mg
H_2O, to a final volume of		100 ml

(D)

Acrylamide	10 g
Bis-acrylamide	2.5 g
H_2O, to a final volume of	100 ml

(E)
Riboflavin (Koch -Light, Art. 4954 h) (4 mg) in H_2O (100 ml)

(F) Prepared fresh weekly
Ammonium persulfate (Serva, Art. 13375) (0.14 g) in H_2O (100 ml)

(G) Bromphenol blue
Bromphenol blue (Merck, Art. 8122) (0.001 g) in H_2O (100 ml)

Annex 2 Solutions for the separating gel and spacer gel.

Separating gel (small pore gel); solutions mentioned below mixed in parts (v/v).

Solution (A)	1 p	total 2 p. When room temperature 20-25°C as it is given. When room temperature is 30°C it is (C) 1.50 p and (C_1) 0.50 p.
Solution (C)	1.75 p	
Solution (C_1)	0.25 p	
H_2O	1 p	

The above mentioned solution is mixed with solution (F) (1:1 v/v).

Note: For isoenzymes of amylase and catalase the H_2O (1 p) was replaced with a 0.6% starch[1]) solution in water (w/v).

Spacer gel (large diameter of pores)

Solution (B)	1 p
Solution (D)	2 p
Solution (E)	1 p
H_2O	4 p.

1) Starch-Hydrolysed. Connaught Medical Research Laboratories, Toronto, Canada (purchased from R. Howell Ltd. Kilburn, High Road, London N.W.6., England).

Annex 3

Fixing solution (LKB, Ampholine PAG plate instructions)

methanol p.a.	150 ml
H_2O	350 ml
sulfosalicylic acid (Merck, Art. 691)	17.25 g
trichloroacetic acid (Merck, Art. 807)	57.5 g

Solution of Coomassie Brilliant Blue R 250 (for staining of proteins in general, L.K.B., PAG plate instructions)

Coomassie Brilliant Blue R 250 (Merck, Art. 12553) (0.115 g) in Destaining solution (100 ml).

Destaining soln.

96% ethanol	500 ml
acetic acid	160 ml
H_2O, to a final volume of	2000 ml

Solution for isoenzymes of esterase

0.2 M Tris-HCl buffer pH 7.4 (2 ml) + Fast Blue RR salt (BDH, Art. 28377/2g) (25 mg) + H_2O (47 ml) is filtered. To this filtered solution is added a 1% solution of α-naphtylacetate in acetone (w/v) (1 ml).

Solution of DAB for isoenzymes of peroxidase

DAB (Fluka AG, Art. 32750)	9 mg
H_2O	25 ml
citric acid/Na_2HPO_4 pH 4.3	25 ml
H_2O_2 (freshly diluted from 30% to 0.6%)	3.5 ml

Solution for isoenzymes of amylase

Iodine (Merck, Art. 4761) (0.004 g), KI (Merck, Art. 5043) (1.5 g) in 0.2 M acetate buffer pH 4.8 (100 ml)

Solution for isoenzymes of catalase

soln. a	0.005 M H_2O_2 in 0.2 M phosphate buffer pH 6.8
Soln. b	saturated soln of Molybdic acid (J.T. Baker, Art. 0176) in H_2O
soln. c	5% KI in H_2O

Electrode buffer soln, pH 8.3

Tris	4 g
Glycine	31 g
H_2O, to a final vol. of	1000 ml