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SPECIFIC DETECTION OF α-AMYLASE ACTIVITY IN CRUDE PLANT EXTRACTS AFTER ISOELECTRIC FOCUSING

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Key Word Index—Procion Red MX 2B amylopectin; α-amylase; β-amylase; isoelectric focusing.

Abstract—A new method which utilizes Procion Red MX 2B amylopectin for the detection of α-amylase in crude plant extracts is described. The substrate is specific only against α-amylase hydrolysis and β-amylase does not attack it. Paper containing Procion Red MX 2B amylopectin applied to gels after isoelectric focusing reveals α-amylase isoenzymes as white bands. When this technique is used, heat-inactivation of β-amylase is not required.

INTRODUCTION

Amylases are involved in the hydrolysis of starch during the germination of seeds. Two distinct types of amylases are present in cereal grains: α-amylase, synthesized *de novo* during the germination process; and β-amylase, present in the starchy endosperm in bound and free forms [1]. Isoenzymes of both α- and β-amylases have been identified and described in the literature [2–5].

It is difficult to assay α-amylase activity in crude plant extracts because of the presence of the β-enzyme; however, it is possible to inactivate malt β-amylase selectively by heating under suitable conditions [6] or alternatively to utilize chromogenic substrates specific for α-amylase [7, 8]. While chromogenic substrates can be utilized for enzymatic determinations of activity, their application to isoelectric focusing is limited because of their low sensitivity when they are included in agar gels and in the film used as an overlay [9].

The incubation of focused gels in starch solutions and staining with iodine represents a specific method for α-amylase detection, only if β-amylase is inactivated by heating of enzyme samples [10]. In this way, one or more unstable α-amylase isoenzymes extracted from some cereal seeds are lost [11, 12]. In addition, such heat treatment cannot be applied to α-amylase determination in other plant tissues (e.g. soybean and alfalfa tissues) in which α-amylase activity is labile [8].

In this paper we describe a method for specific detection of α-amylase isoenzymes after isoelectric focusing without previous heat-inactivation of β-amylase. The method utilizes a soluble chromogenic and very sensitive substrate, Procion Red MX 2B amylopectin, previously used in clinical [13] and forensic science [9].

RESULTS AND DISCUSSION

Sax *et al.* [13] demonstrated that Procion Red MX 2B amylopectin is a soluble and very sensitive substrate for human salivary amylase, but they did not consider its specificity against β-amylase hydrolysis because animals have no β-amylase. We tested the substrate with commercial β-amylase using from 1 to 3000 U: no coloured soluble sugars were released from the red-dyed amylopectin. On the contrary, the substrate is specific and very sensitive (< 0.5 U) against α-amylase hydrolysis. This property of the synthetic substrate can be easily utilized for α-amylase assay in the presence of β-amylase. In particular, the solubility of Procion Red MX 2B amylopectin allows paper sheets to be made on which the substrate can be precipitated. The pink paper can then be

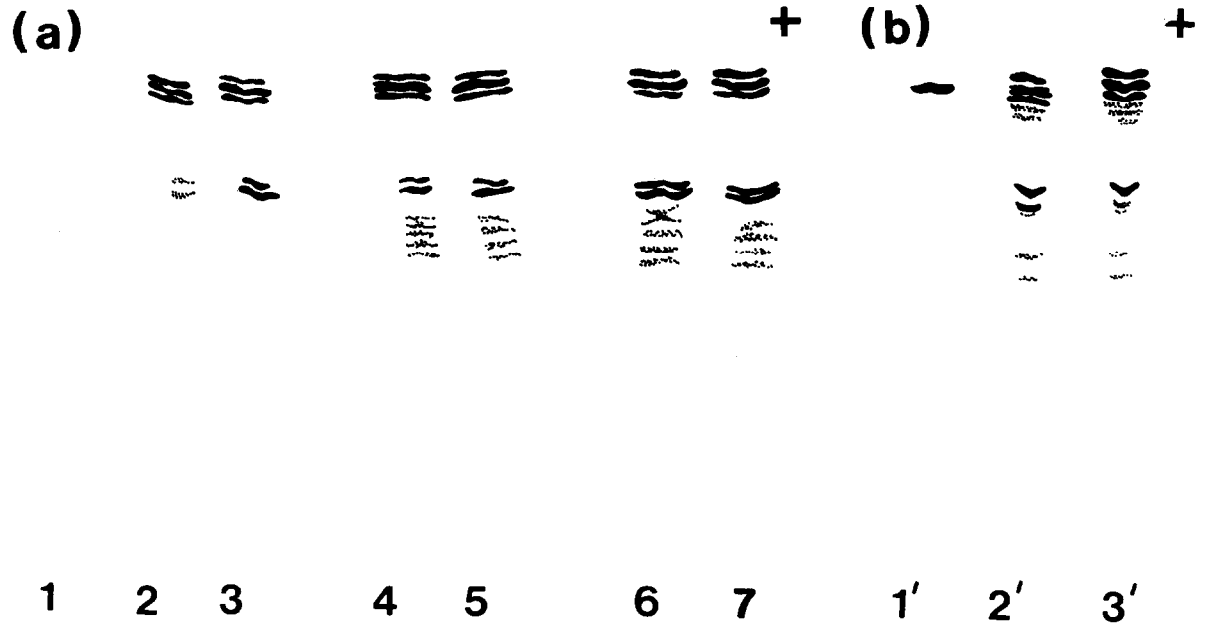

Fig. 1. (a) Zymogram of α-amylase activity using sensitive paper. Samples: (1) β-amylase (1500 U); (2, 4, 6) heated and (3, 5, 7) untreated (unheated) preparations from *T. durum* endosperms (0.5, 1 and 2 U, respectively). (b) Detection of protein bands by Coomassie Brilliant Blue R-250. Samples: (1′) β-amylase; (2′ and 3′) proteins of heated and untreated (unheated) preparations from *T. durum* endosperms.

used for the detection of α-amylase isoenzymes after isoelectric focusing. Figure 1a shows the pattern of α-amylase isoenzymes extracted from *Triticum durum* seeds and Fig. 1b shows the detection of protein bands of β-amylase. There are two groups of isoenzymes: group I with a pI of 6.0–6.8 and group II with a pI of 4.5–4.8. This pattern is similar to that found by Sargeant and Walker [10] in wheat, using heat-inactivation of samples and a starch–iodine method to develop the activity bands after isoelectric focusing. In our preparations, the more active isoenzymes had a pI of 4.5–4.8, while the others with a pI of 6.0–6.8 had two components more active and at least four components with less activity. This was demonstrated by applying differential concentrations of enzyme on the plate or using different incubation times to develop the paper. For samples extracted from *Triticum durum* endosperms, no differences were observed between heated and untreated preparations, but we think that these data cannot be generalized because in some seeds heat-inactivation can cause the loss of some activity band [11, 12]. The identical pattern of heated and untreated samples further confirms the specificity of the substrate against α-amylase hydrolysis because most of the interfering enzymes are destroyed during heat treatment [6].

In conclusion, the method appears specific, sensitive and useful for application to the detection of α-amylase isoenzymes in crude plant extracts. The possibility of direct application of such extracts on isoelectric focusing plates without previous inactivation of β-amylase prevents losses of labile α-amylase forms and makes the method particularly useful for those plant tissues to which heat treatment cannot be applied [8].

EXPERIMENTAL

Enzyme sources. Commercial sweet potato β-amylase (Sigma) was utilized. Amylase activity was extracted from 30 endosperms of *Triticum durum* cv Cappelli seeds (1982: stored under laboratory conditions) previously sterilized and germinated for 7 days at 23° in the dark. The endosperms were homogenized in 6 ml 0.1 mol/l. NaOAc buffer (pH 5.5) containing 6 mmol/l. $CaCl_2$ in a mortar, centrifuged at 3000 *g* and the supernatant was desalted using small Sephadex G-25 columns [14]. Heat treatment of enzyme samples was performed as suggested in ref. [6].

Synthesis of Procion Red MX 2B amylopectin. This was performed according to ref. [15] for dyeing of polysaccharides. Amylopectin and Procion Red MX 2B were purchased from Serva Feinbiochemica.

Preparation of sensitive Procion Red MX 2B amylopectin paper. This was made by a modification of the method of ref. [9]. Procion Red MX 2B amylopectin (0.6% w/v) was dissolved in 0.15 mol/l. NaOAc buffer containing 6 mmol/l. $CaCl_2$, and Whatman No. 3 paper sheets were saturated by dipping them in the soln. The paper was allowed to dry and sprayed with Me cellosolve (undiluted). The pink paper sheets were stored at room temp. wrapped in aluminium foil.

Spectrophotometric assay of β-amylase. To 1 ml of Procion Red MX 2B amylopectin was added an appropriate amount of β-amylase (1–3000 U) and the soln was incubated at 30°. At the end of the incubation period, the reaction was blocked by addition of 5 ml Me cellosolve and 0.2 ml $ZnSO_4$ soln (100 g/l). The solns were centrifuged in a bench centrifuge and the *A* of the supernatants read at 517 nm against a control in which the reaction was blocked before incubation. Commercial β-amylase was also assayed using Bernfeld's method [16].

Isoelectric focusing experiments. Isoelectric focusing of the

samples was performed with a LKB-Multiphor apparatus. Gels (0.5 mm thick) containing carrier ampholytes in the pH range 3.5–9.5 were utilized. Samples were applied using filter paper wicks. A constant power of 0.8 W per cm length of gel was passed through the gel at 3° for 2 hr. Gels were prefocused for 30 min. At the end of the experiment, the pink paper was pressed gently onto the gel and the white bands of enzymatic activity were visually discernible in 5–10 min, depending upon the enzymatic activity used. The reaction was interrupted by flooding the gel and paper in 1 mol/l. HCl soln. The paper was removed carefully from the gel and allowed to dry. The gel was fixed and stained with Coomassie Brilliant Blue R-250 for protein detection.

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CHEMICAL VARIATIONS OF *ASAHINEA CHRYSANTHA*

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Key Word Index—*Asahinea chrysantha*; Parmeliaceae; lichen; α- and β-collatolic acids; haematommic acid.

Abstract—A sample of the lichen *Asahinea chrysantha* collected in the northern region of the Soviet Far East contained α- and β-alectoronic acids, while the same lichen collected in the southern region contained the methoxy derivatives, α- and β-collatolic acids, of these two acids.

We recently reported on the chemical composition of a sample of the lichen *Asahinea chrysantha* (Tuck.) Culb. & Culb. collected in the Magadan district (63°N., 1000 m, on granite, sample I) [1, 2].

We have now investigated a sample of the same lichen from the Primorskiy district (44°N., 500 m, on ryolite, sample II). It had a grey upper surface whereas that of sample I was bright yellow. Other differences of the basic thallus structure could not be found (voucher specimens deposited at the herbarium of the Tartu State University).

The chloroform extract of sample II was chromatographed over Sephadex LH-20 and nine compounds were isolated (see Table 1). Compounds **1**, **2**, **5**–**7** and **9**–**10*** were identified by direct comparison with authentic samples (mmp, TLC, MS and NMR). The ninth compound (**11**) was identical to 1,4,5,6,8-pentahydroxy-3-methylanthraquinone [MS: *m/z* 302 $[M]^+$, 286, 274; UV λ_{max}^{EtOH} nm: 228, 261, 302; R_f (TLC)]. Its presence in *A. chrysantha* ([1] and this report) and in *A. scholanderi* (Llano) Culb. & Culb. [3] suggests that this pentahydroxymethyl anthraquinone is typical for the genus *Asahinea* and hence we have named it asahinin. There was no possibility of studying the chemical composition of the third species of the genus *Asahinea*, *A. kurodakensis* (Asah.) Culb. & Culb., which contains lavender pigments as well as the other two species [4].

Sample II contained haematommic acid, [MS *m/z*: 196 $[M]^+$, 178 $[M-H_2O]^+$, 168 $[M-CHO]^+$, 152 $[M-CO_2]^+$; ^1H NMR ($CDCl_3$): δ2.60 (3H, *s*, Me), 6.35 (H, *s*), 10.35 (H, *s*, CHO), 12.53 (H, *s*, OH)], a compound that has not been reported before as a natural lichen component [5].

Table 1 shows that sample I contained α- and β-alectoronic acids, while sample II contained the methoxy derivatives of these acids. This is the second report of the

*Structures not shown.