

Marcella Chiari
Arianna Gelain
Sergio Riva
Debora Tura

Istituto di Chimica degli Ormoni,
Milano, Italy

Analysis of mandelonitrile lyase and β -glucosidase from sweet almonds by combined electrophoretic techniques

Almonds are a rich source of mandelonitrile lyase (oxynitrilase) and β -glucosidase. The isolation of these two enzymes from sweet almonds requires fractional ammonium sulfate precipitation followed by ion-exchange chromatography on diethylaminoethyl-(DEAE) and carboxymethylcellulose (CMC) columns. In the present investigation different electrophoretic techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing in immobilized pH gradients (IEF-IPG), and capillary electrophoresis were used to characterize these two enzymes. For the first time, β -glucosidase and oxynitrilase were separated in an immobilized pH gradient of one pH unit. Capillary zone electrophoresis (CZE) was an excellent tool for analysis of the purity of enzyme preparations, achieving complete separation of various protein constituents in only 15 min. CZE showed a resolving capacity for the separation of enzyme forms comparable to that of isoelectric focusing in an immobilized pH gradient.

1 Introduction

An extract of defatted almond meal contains the enzymes β -glucosidase and mandelonitrile lyase among others. Hydroxynitrile lyases, also called oxynitrilases, catalyse the formation of a chemical equilibrium between cyanohydrines and their corresponding aldehydes (or ketones) and HCN [1]. Specifically, in the presence of excess hydrogen cyanide, the oxynitrilase from almonds catalyses the stereoselective addition of HCN to several aromatic and aliphatic aldehydes to form *R*-cyanohydrines which are useful building blocks for asymmetric syntheses [2, 3]. The first attempts to exploit this enzyme date back to 1963, when Becker and Pfeil [1] extracted oxynitrilase from almonds with the aim of immobilizing it on a cellulose column to catalyze the production of optically active (*R*)-mandelonitrile. The properties of this enzyme were systematically investigated three years later by Seely *et al.* [4] who proposed a purification protocol which gave an enzymatic preparation 250 times purer than the initial extract. This procedure is currently used, with slight modifications, for the production of homogeneous enzyme samples. Four different isoforms of this enzyme, all consisting of a single polypeptide chain with a molecular mass of approximately 60 kDa, have been described [3, 5]. The existence of isoenzymes is of interest, as each isoform might have its own substrate specificity. In a recent investigation Smitskamp-Wilms *et al.* [3] showed there were four isoforms of oxynitrilase, two of them accounting for over 90% of the whole oxynitrilase content. The two main isoforms were separated by fast protein liquid chromatography (FPLC) on an anion exchange column. However, although different

authors agree on the existence of the four isoforms, unequivocal data on their isoelectric points have not been reported [3, 6].

The properties of β -glucosidase, the other enzyme with importance for synthesis found in almond extract, are even less clear. *In vivo* glycosidases catalyze the hydrolysis of glycosidic bonds, while *in vitro* their action can be reversed to catalyze the stereospecific formation of glycosides of unprotected sugars [7]. β -Glucosidase is a glycoprotein made up of two 65 000 Da subunits whose characterization is hindered by the microheterogeneity of the enzymatic extract [8–10]. It has been purified to various degrees, and the different preparations have dual activity, being able to hydrolyze β -D-glucopyranosides as well as β -D-galactopyranosides [9, 11]. It has still not been clearly demonstrated whether the dual activity is due to different proteins or derives from a bifunctional character of this biocatalyst. Although some of these isoenzymes have been isolated by ion exchange chromatography and by preparative isoelectric focusing in Ampholine [9], the information on their properties is still incomplete and in some cases not consistent with the purification procedure adopted. The existence of a certain number of isoforms – at least four – with different *pI* has been demonstrated by several authors [8, 9], but the *pI* of only one of these (*pI* 7.0) has been reported. Both oxynitrilase and β -glucosidase are currently used for synthetic applications in our laboratory. We are using β -glucosidase to glucosylate various double-bond-containing alcohols, with the aim of producing monomers that can be polymerized to give hydrophilic polymeric coatings [12] for capillary electrophoresis and DNA separation matrices. The isolation of these two enzymes from sweet almonds requires fractional ammonium sulfate precipitation of the proteins extracted from the defatted almond meal, followed by ion exchange chromatography on diethylaminoethyl- (DEAE) and carboxymethylcellulose (CMC) columns [3, 4]. This article provides information on the isoelectric point of the isoforms of these two enzymes recovered from the different purification steps. For the first time *pI* values of oxynitrilase and β -glucosidase isoforms have been deter-

Correspondence: Dr. Marcella Chiari, Istituto di Chimica degli Ormoni, Via Mario Bianco 9, 20131 Milan, Italy (Tel: +02-2847737; Fax: +02-2841934; E-mail: ico@siam.mi.cnr.it)

Nonstandard abbreviations: CMC, carboxymethylcellulose; CZE, capillary zone electrophoresis; DEAE, diethylaminoethyl; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid)

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mined by isoelectric focusing in immobilized pH gradients (IEF-IPG). The purity of these enzymes was also investigated by capillary zone electrophoresis (CZE), which was an excellent tool for assessing cross-contamination and for characterizing the different enzyme forms.

2 Materials and methods

2.1 Chemicals

Tris(hydroxymethyl)aminomethane (Tris), *N,N*-bis(2-hydroxyethyl)glycine (Bicine), piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), acetic acid, ethylenediaminetetraacetic acid (EDTA), ammonium sulfate, biuret reagent, Folin-Ciocalteu phenol reagent, *N,N*-dimethylformamide, β -glucosidase from almond, mandelonitrile lyase from almond, DL-mandelonitrile and *p*-nitrophenyl β -D-glucopyranoside were purchased from Sigma Chemical Company (St. Louis, MO, USA). Acrylamide, *N,N'*-methylenebisacrylamide, ammonium peroxydisulfate, *N,N,N',N'*-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate and SDS protein markers were from Bio-Rad Labs (Hercules, CA, USA). Immobiline buffers, GelBond PAG and Pharmalyte 3–10 and *pI* protein markers were from Pharmacia-LKB (Uppsala, Sweden). *N*-acryloylaminoethoxyethanol (AAEE) was synthesized according to Chiari *et al.* [13]. Ion exchange resins DEAE EMD-60 (M) and CMC were from Merck (Darmstadt, Germany).

2.2 Apparatus

Multiphor II, Macrodrive power supply ECPS 3000/1150 Multitemp thermostat were from Pharmacia-LKB. Mini Protean II Electrophoresis System and Power-PAC power supply 300 were from Bio-Rad. CZE separations were performed in a Spectra-Phoresis 1000™ capillary system (Thermo Separation Products, Fremont, CA, USA). Data were collected on a personal computer using SW-Phoresis 1000 software. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were coated as described in [14]. HPLC separations were carried out with a Jasco 880 PV pump, equipped with a Jasco 870 UV detector and a Jasco V-530 spectrophotometer (Jasco, Tokyo, Japan).

2.3 Enzyme extraction

Almond (*Prunus amygdalus*) seeds (1 kg) were ground and defatted by continuous extraction with ethyl acetate in a Soxhlet apparatus. The remaining powder was suspended in a 10 mM solution of ammonium hydroxide (70 mL for 10 g of powder) and shaken overnight. Following equilibration to pH 5.4 with 1 M H_3PO_4 and centrifugation at 6000 g for 30 min, the supernatant was subjected to fractional precipitation in 40% and 60% ammonium sulfate.

2.4 Enzyme purification

The precipitate obtained from 60% ammonium sulfate saturation was dialyzed against 20 mM phosphate buffer,

pH 6.3 (buffer A) and loaded on a DEAE EMD-650 Fractogel column (10 × 3.5 cm, ID) previously equilibrated with the same buffer. Most of the β -glucosidase was not retained by the column and was eluted in the void volume, whereas oxynitrilase was eluted by applying a linear gradient of NaCl (0–500 mM NaCl) in the interval from 75 to 100 mM NaCl. The peak fractions with oxynitrilase activity were pooled and dialyzed against water (sample B). The solution with glucosidase activity (fraction A) was treated with ammonium sulfate up to 60% and stirred at room temperature for 1 h. The precipitate, containing β -glucosidase, was recovered, dissolved and dialyzed against 50 mM acetate buffer, pH 3.6 (buffer B). The solution was loaded on a CMC column equilibrated with buffer B. The various β -glucosidase forms were eluted from the column by applying a linear gradient of pH and ionic strength (from 50 mM sodium acetate buffer, pH 3.6, to 500 mM sodium acetate, pH 4.9). The three peak fractions showing glucosidase activity were pooled separately (fractions C, D, E). Fraction C was eluted in the interval from 0.13–0.15 M AcONa and pH 3.68–3.70; fraction D: 0.17–0.24 M AcONa, pH 3.73–3.82; fraction E: from 0.26–0.34 M AcONa and pH 3.8–4.01.

2.5 Protein estimation and enzyme assay

The protein content of the solution was estimated either by measuring absorbance at 280 nm or by the method of Lowry *et al.* [15]. β -Glucosidase assays were carried out by monitoring the increase in absorbance at 400 nm due to the appearance of *p*-nitrophenolate (yellow) produced by the enzyme-catalyzed hydrolysis of *p*-nitrophenyl β -D-glucopyranoside. The assay solution contained 20 mM of the glucoside in 50 mM sodium acetate, pH 6.0. The assay was performed at room temperature. The extinction coefficient used in the calculations was $3.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. Oxynitrilase activity was assayed by monitoring the increase of absorbance at 249 nm due to the benzaldehyde released by the enzymatic hydrolysis of DL-mandelonitrile. Solutions containing 990 μL of 100 mM citrate buffer, pH 5.5, and 3 μL of a 2.4% v/v solution of DL-mandelonitrile in dimethylformamide were used. The extinction coefficient of benzaldehyde is $13.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ [3].

2.6 Electrophoretic separations

2.6.1 SDS-PAGE

SDS-electrophoresis was performed on a MINI-Protean II dual slab cell. The gel was 7 cm long, 8 cm wide and 1 mm thick. The separations were performed on 10–20%T, 2.6%C polyacrylamide-gradient SDS gels and under the discontinuous conditions of Laemmli [16]. Broad-range and low-molecular-mass standards from Bio-Rad were used to extrapolate the molecular mass of the enzymes.

2.6.2 IEF-IPG

IEF in IPG was performed in a pH 4–8 and in a pH 3.8–4.8 range [17]. After casting and polymerization, the

gels were extensively washed in distilled water, air-dried, and then rehydrated in water. The protein content of all the samples was estimated by the method of Lowry. Twenty microliters of sample, diluted as to contain 1 mg of protein, were loaded in sample cups at the cathodic gel side. Running conditions were: 2 h at 400 V followed by 6 h at 2000 V, 10°C. The gels were stained either with Coomassie Brilliant Blue R-250 in Cu^{++} [18], or, to detect β -glucosidase activity, by placing them in a freshly prepared solution of 0.05% β -naphthyl- β -D-glucopyranoside, 0.03% Fast Blue RR salt and 1% acetone in water. After 30 min, the bands corresponding to glucosidase forms developed a red color and the gel was rinsed for 5 min with a 1% glycerol solution.

2.6.3 CZE

CZE of the DEAE and CM cellulose fractions was performed in a poly(acryloylaminoethoxyethanol)-coated capillary [14]. The separations were carried out in a 50 μm ID capillary, 44 cm long, 37 cm to the detector. Sample solutions were injected for 1 s by hydrodynamic injection and the detector was set at 214 nm. The temperature was set at 25°C. Running conditions: 25 mM Bicine-Tris buffer, pH 8.5, 30 kV, anodic migration.

3 Results and discussion

Oxynitrilase and β -glucosidase are currently used in our laboratory as biocatalysts for the synthesis of different products and, according to published suggestions [3], we have developed an efficient procedure for obtaining relatively large amounts of these two enzymes from ground almonds. The precipitate obtained from a fractional ammonium sulfate precipitation was purified by chromatography on a DEAE column (see Section 2.4). β -Glucosidase did not interact with the resin at the operative pH (20 mM phosphate buffer, pH 6.3) and was mainly eluted in the void volume (fraction A). Oxynitrilase (fraction B) was eluted in a narrow NaCl concentration range (75–100 mM) and was detectable with the naked eye due to the presence of one FAD molecule as prosthetic group. This enzyme was recovered in a 67% yield with a specific activity of 101 U/mg (purification factor, 4.0).

The so-called "fraction A" was separated in three different fractions (C, D, E) by CMC ion exchange chromatography. Table 1 summarizes the results of the purification of β -glucosidase.

As discussed below, several electrophoretic techniques have been adopted to characterize the two enzymes and to assess the purity of the various chromatographic fractions. The purified oxynitrilase preparation (fraction B) and the fractions with glucosidase activity recovered from CMC chromatography (fraction A) were initially subjected to SDS-PAGE under reducing conditions and compared with commercial preparations of β -glucosidase and oxynitrilase. Only one major band with R_f 0.7 was observed for oxynitrilase (sample B), showing a molecular mass of 63 kDa in agreement with the value reported in the literature [8, 9]. One of the three fractions eluted from the CMC column (fraction C) presented a single band with a relative mobility of 0.7, and a molecular mass of 65 kDa, which agrees with the molecular mass reported for the β -glucosidase subunit [8]. Fractions D and E contained a minor component with a relative mobility corresponding to the band of fraction C and major bands with higher relative mobility. It was not possible to distinguish β -glucosidase from oxynitrilase by SDS-PAGE under reducing conditions, the molecular mass of the latter protein coinciding with the molecular mass of the β -glucosidase subunits.

Our enzymatic samples (fractions B, C, D, and E) were examined by IEF-IPG, with the aim of determining the isoelectric points of the various isoforms. This technique offers an extremely high resolving power as narrow pH gradients can be generated (one pH unit) that allow discrimination of isoforms with pI differences less than 0.01 unit. An IEF-IPG gel in the pH range 4–8 was stained for protein with Coomassie Blue and for β -glucosidase activity as described in Section 2.6.2. In one of the CMC-eluted fractions (fraction C), all bands revealed by Coomassie Blue showed β -glucosidase activity, while mixtures of β -glucosidases and other protein contaminants were present in samples D and E. Table 2 summarizes the pI values of various β -glucosidase forms. Values ranged from 4.4 to 4.6 in fraction C, while fraction D contained active forms of glucosidase with pI from 5.4 to

Table 1. Purification of β -glucosidase from almond

Sample	Volume mL	Total activity U	Proteins mg	Specific activity U/mg	Yield %	Purification factor
Crude extract						
	1.600	6.500	4.800	1.4	100	1.0
Supernatant at 40% $(\text{NH}_4)_2\text{SO}_4$	1.800	5.200	2.800	1.9	80	1.4
Precipitate at 60% $(\text{NH}_4)_2\text{SO}_4$	174	4.400	1.400	2.9	68	2.1
Fraction A from DEAE	95	4.250	1.000	3.9	65	2.8
Total activity recovered from CMC	304	4.200	620	6.8	64	4.9
Fraction C	36	530	10	53.0	8	37.9
Fraction D	104	2.750	260	10.6	42	7.6
Fraction E	164	920	350	2.6	14	1.9

Table 2. Isoelectric point of β -glucosidase and oxynitrilase isoforms

Enzyme	pI ^{a)}
β -Glucosidase	
fraction C	4.4–4.6
fraction D	4.5–5.6, 6.0–7.0
Oxynitrilase	
	4.32, 4.34 (major bands)
	4.26, 4.42 (minor bands)

a) Determined at 10°C

5.6 and from 6.0 to 7.0. In the same pH range (4–8), oxynitrilase presented a series of not completely resolved bands with a pI around 4.4.

Despite the excellent resolving power of this technique, it was not possible to evaluate cross-contamination between oxynitrilase and β -glucosidase as the acidic forms of the latter have the same pI as oxynitrilase. A closer inspection was therefore made in a narrow IEF-IPG gel of one pH unit (pH 3.8–4.8; Fig. 1). The purest β -glucosidase preparation (sample C) contained a protein with no enzymatic activity, with a pI corresponding to one of the bands in the oxynitrilase sample. In this pH range complete separation of the various forms of both enzymes was achieved. The specific staining test for β -glucosidase allowed the identification of four groups of enzymatic bands, all containing several forms with pI 4.0–4.8. Table 2 shows the pI values of different oxynitrilase forms. For instance, in the commercial enzyme there are six bands with pIs from 4.26–4.42. The two major bands showed a pI of 4.32 and 4.34. The high resolving power of IEF-IPG in a narrow pH range provides an efficient tool for analysis of the isoforms of β -glucosidase and oxynitrilase. However, the technique is time-consuming and requires specific skills. On the other hand, CZE, with its high resolving power, automation and quantitative capacity, is becoming an important tool in the analysis of proteins [19]. Thanks to the introduction of new types of silica polymer coatings that suppress protein wall interactions, it is now possible to achieve the high efficiency in the separation of macromolecules predicted by theory.

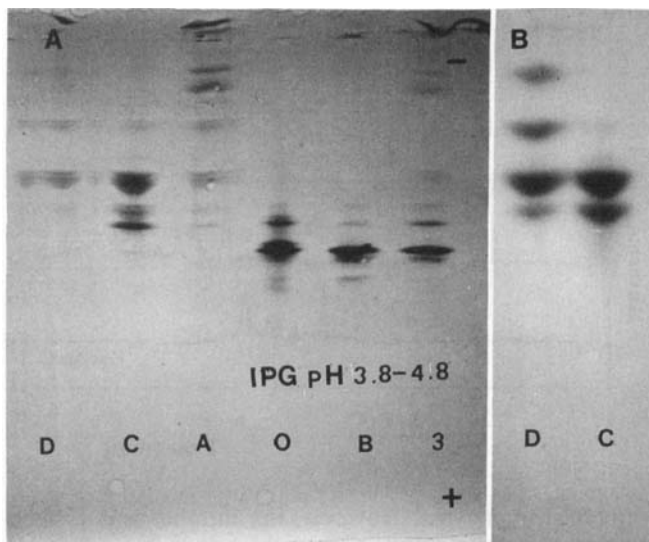


Figure 1. IEF of oxynitrilase and β -glucosidase in a pH 3.8–4.8 IPG. (A) Staining with Coomassie, (B) staining for β -glucosidase activity. The focusing conditions are described in Section 2.6.2. The samples are applied at the anodic side. Samples from left to right: D, fraction D; C, fraction C; A, fraction A; O, commercial oxynitrilase; B, fraction B; 3, crude almond extract.

In our hands CZE proved to be an excellent tool for the separation of oxynitrilase from β -glucosidase and for the analysis of their forms. Separations were performed in a capillary coated with poly(N-acryloylaminoethoxyethanol) according to [14]. Figure 2 shows three electropherograms corresponding to the crude almond extract (fraction 3), the sample unretained on the DEAE column (fraction A) and the oxynitrilase eluted from the same column (fraction B). The separation was completed in 15 min and did not require any cumbersome gel-gradient casting procedure. The electropherograms in Fig. 3 show that all the components of crude almond extract were eliminated from the oxynitrilase preparation. Fraction A, tested for β -glucosidase activity, was found to

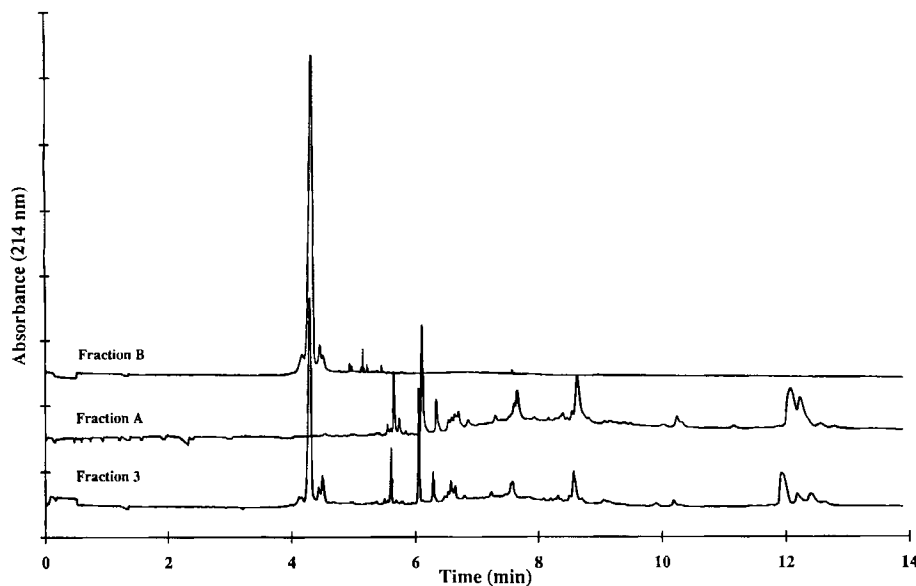


Figure 2. Electropherograms of the crude almond extract (fraction 3), of the sample unretained on the DEAE column (fraction A) and of the oxynitrilase eluted from the same column (fraction B). The separations were carried out in 50 μ m ID capillary, 44 cm long (37 cm to the window) coated with poly(acryloylaminoethoxyethanol) in 25 mM Bicine-Tris buffer, pH 8.5. Running conditions: 30 kV, anodic migration.

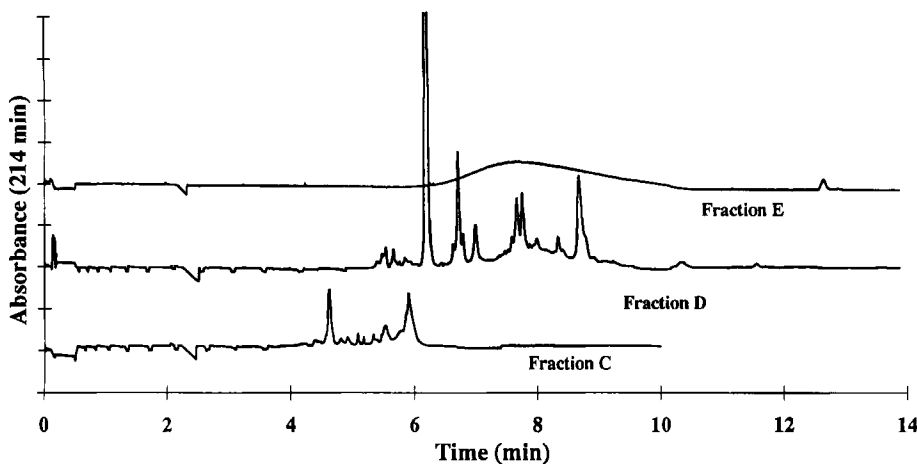


Figure 3. Electropherograms of three different purification fractions (fractions C, D, E) of β -glucosidase obtained from a CMC-cellulose column. Separation conditions as in Fig. 2.

contain the enzyme. However, the number and concentration of contaminants were so high that it was impossible to distinguish β -glucosidase peaks in the electropherogram. The highest β -glucosidase-specific activity was in fraction C following the CMC chromatography. The electropherograms presented in Fig. 2 shows the wide microheterogeneity of β -glucosidase. Although fractions D and E contained β -glucosidase forms, these were only minor components that could not be resolved from the bulk of the contaminants in the separation conditions adopted.

4 Concluding remarks

The aim of this work was to characterize two almond enzymes, oxynitrilase and β -glucosidase, by different electrophoretic techniques: SDS-PAGE, IEF-IPG and CZE. For the first time, β -glucosidase forms were separated according to their *pI* in an IPG of one pH unit. CZE proved an excellent tool for analyzing the purity of enzyme preparations, allowing complete separation of various protein constituents in only 15 min. CZE also had a resolving capacity in the separation of enzyme forms comparable to that of isoelectric focusing in immobilized pH gradient, which is one of the most powerful techniques for resolving proteins according to their *pI*. Compared to IPG gels, CZE has the advantage of being fully automated and less time-consuming.

5 References

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