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A simple & Rapid Method For Detecting Bacterial Myrosinase Corresponding Protein Band

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ABSTRACT

Myrosinases have significant scientific and medical implications. Unfortunately, detection and purification of myrosinase from microbes requires the use of highly cost substrates (glucosinolates) such as sinigrin and expensive instruments such as Fast Protein Liquid Chromatography and or ion exchange chromatography. In this work, we used only 20 mL of bacterial culture supplemented with sinigrin (10 mM) to obtain partially purified myrosinase. The crude protein extract was loaded onto native polyacrylamide gel and putative myrosinase band was identified and eluted. This step successfully minimised the numbers of protein bands of bacterial crude extracts to be further analysed. The current method describes a simple, rapid and cost effective protocol for isolation and detection of active bacterial myrosinases. Furthermore, our method can be used as a purification step.

طريقة سهلة وسريعة للكشف عن إنزيم المايروسينيز البكتيري وتحديد حزمة البروتين المسؤولة عن وجوده

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الكلمات المفتاحية:
انزيم المايروسينيز
الكشف
سنيجرين
بولي أكريلاميد الأصلي

الملخص

لانزيمات المايروسيناز أهمية علمية وطبية كبيرة. ولسوء الحظ فإن طرق الكشف وتنقية المايروسينيز من الميكروبات يتطلب استخدام مواد كيميائية عالية التكلفة (الجلوكوزينات) مثل سينيجرين وإستخدام أجهزة باهظة الثمن مثل كروماتوجرافيا السائل لتنقية البروتين (FPLC) أو كروماتوجرافيا التبادل الأيوني. في هذه الدراسة، استخدمنا فقط 20 مل من المزرعة البكتيرية مدعمة بالسنيجرين (10 ملي مول) وتمّ الحصول على أنزيم المايروسينيز المنقى جزئيا. تم تحميل مستخلص البروتين الخام على هلام (جل) بولي أكريلاميد الأصلي وتم التعرف على الحزمة البروتينية الدالة على هذا الأنزيم. أدت هذه الخطوة بنجاح إلى التقليل من أعداد الحزم البروتينية من البروتين البكتيري الخام المستخدم ليتم تحليلها. تصف الطريقة الحالية خطوات بسيطة وسريعة وفعّالة من حيث التكلفة لعزل واكتشاف أنزيم المايروسينيز البكتيري الفعّال. علاوة على ذلك يمكن استخدام طريقتنا كخطوة لتنقية هذا البروتين (المايروسينيز).

Introduction

β-glucosidases represent a major group of enzymes among glycoside hydrolases. Plant β-glucosidases catalyse the hydrolysis of substrates

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with a *β-O*-glucosidic linkages and this type of enzyme is placed in glucohydrolase family 1 (GH1) (*β*-D-glucoside glucohydrolase, EC 3.2.1.21). Other types of plant *β*-glucosidases, hydrolyse *S*-linked *β*-glycosidic bonds (e.g. glucosinolates) that are also placed in GH1 (*β*-D-thioglucoside glucohydrolase, EC 3.2.3.1) and known by the trivial name myrosinase; [4,9,10,12]. Myrosinase was first discovered in mustard seed in 1840 [5] , is also found in certain intestinal bacteria *Enterobacter cloacae* [25] and *Paracolobactrum aerogenoides* [17], *Lactobacillus agilis* strain R16 [19], *Bifidobacterium* (*B. pseudocatenulatum; B. adolescentis; B. longum*) [6], *Bacteroides thetaiotaomicron* [8], *Bacteroides vulgatus* [20], *Enterococcus casseliflavus* CP1, *Escherichia coli* VL8 [15]) and also found in soil isolate *Citrobacter* WYE1 [1], fungi *Aspergillus sydowi* [22] and *Aspergillus niger* [18], cruciferous aphids *Brevicoryne brassicae* and *Lipaphis erisimi* [2].

Myrosinases are responsible for the conversion of glucosinolates into anti-carcinogenic or anti-cancer compounds known as isothicynates [11,13,16,21,24]. Therefore, myrosinases have been extensively studied however very few bacterial myrosinases have been reported [1,7] since they are mostly inactive in vitro [15,19]. Two methods are commonly used for bacterial myrosinase activity detection; the first is growing the bacteria and extraction of the proteins in order to assay myrosinase activity. The second method is detection of isothiocynates formed from incubation of glucosinolate substrate by intact bacterial cells or their cell free extract for myrosinase activity. The latter requires expensive equipment such as gas chromatography (GC) and high performance liquid chromatography (HPLC). Both methods cannot be used in identification of protein responsible for myrosinase activity especially if the organism is a new isolate and no reference in the genome library. In this study we propose a simple and rapid protocol that can be followed in any lab not for only myrosinase detection but also for peptide purification and subsequent identification.. Kwon et al. [14] reported the method for the detection of *β*-glucosidases using esculin substrate. Myrosinase activity of plant origin was detected by incubating isoelectric focusing (IEF) gel loaded with protein extract with barium sulphate [3] however active myrosinase was not eluted from the gel. Zymogram technique was used to detect a number of enzymes including *β*-glucosidase from maize using SDS PAGE [10]. Barium chloride agar was reported for detection of myrosinase producing fungi [23]. None of these methods described the isolation of active myrosinase. In the present study we present a simple and rapid protocol to screen, elute and purify bacterial myrosinase using polyacrylamide gel electrophoresis (PAGE).

Materials & Methods

Growth condition & crude protein extraction

Citrobacter WYE1 known to have an inducible myrosinase activity [1] was used in this study. The test organism was grown aerobically (37 °C, 24 h) on 20 mL M9 liquid medium containing the followings per litre ($Na_2HPO_4.7H2O$ (12.8 g), KH_2PO_4 (3.1 g), NaCl (0.5 g), NH_4Cl (1.0 g), $MgSO_4.7H_2O$ (0.5 g) plus sinigrin (10 mM) which was replaced by glucose in the control medium. The cells were pelleted (4000 rpm, 15 min, 4 °C) and washed twice with citrate phosphate buffer (CPB) (20 mM, pH 6.0). The cell free protein extract was obtained from 5 mL cell suspension in CPB by a single shot cell disruptor (Constant System ltd., Northants, UK) followed by centrifugation (10000xg, 15 min, 4 °C) to remove cell debris. Clear supernatant containing proteins was desalted using Econo-Pac 10DG desalting Columns (BIORAD, USA) with CPB as exchange buffer [1] The desalted crude protein extract was sterilised using 0.22 µm filter membrane and used in all conducted tests. Protein concentration was determined by Bradford reagent (Bio-Rad, UK) and myrosinase activity was determined by GOD PERID method (Huggett and Nixon 1957).

Preparation of polyacrylamide gel electrophoresis

Both SDS-PAGE (8%) and non denatured native PAGE (8%) were prepared as described by Bollag and Edelstien (1991). For native gel electrophoresis; the protein crude extract of induced cells and the control (non-induced) were mixed with sample buffer (Tris HCl 0.312 M, glycerol 50% and bromophenol blue 0.05%) and loaded into gel wells. Electrophoresis buffer (Tris HCl 25 mM, glycine 192 mM)/L^{-1} was poured into gel tank. The gel was run (140 V / 90 min) but with the gel rig in an ice bath. SDS gel was carried out as mentioned in [1]. Internal peptide sequencing of SDS PAGE band of 66 kDa was carried out as mentioned by [1].

Elution of bacterial myrosinase

A native PAGE was used for this experiment. Following electrophoresis the gel was washed twice (10 min each) with CPB (20 mM, pH 6.0) in order to exchange buffer. After the last wash the gel was incubated (25 °C) with 2 mL of esculin reagent (esculin 0.1% and ferric chloride 0.03%) or with 1 mM *p*-nitrophenyl-*β*-D-glucopyranoside (*p*NPG). The protein bands representing *β-O*-glucosidase activity were observed by the appearance of dark brown band as a resulting end product of esculin hydrolysed by *β-O*-glucosidase and yellow band as a resulting end product of PNPG hydrolysis. These bands then were excised from the gels. These gels were carried out in triplicates. One selected active band from one gel was incubated (25 °C, 1 h) with sinigrin (2 mM) in CPB (20 mM. pH 6.0) in order to confirm myrosinase activity. The remaining active bands were used for protein elution. These bands were minced into small pieces and incubated in 4 mL of CPB (20 mM, pH 6) and incubated overnight (4 °C) and briefly centrifuged in order to pellet the gel pieces. The supernatant containing eluted myrosinase was carefully transferred into a fresh falcon tube and then desalted against the same buffer on an Econo-Pac 10DG desalting Columns (BIORAD, USA). Myrosinase activity was detected by GOD PERID method as previously described [1].

The eluted protein was subjected to SDS-PAGE in order to check purity and molecular weight. Prior to electrophoresis the eluted protein was concentrated to 1 mL using ultra filtration membrane tube NMWL 10.000 (4 mL UFC801024, Amicon ultra, Millipore, USA). An aliquot (22 µL) of the concentrated protein was combined with sample buffer (4X, 7.5 µL). Then, the mixture was boiled (3 min) and loaded into SDS-PAGE using. Low molecular weight protein marker (Sigma, USA).was also applied into the gel. Following electrophoresis the gel was stained with Coomassie instant blue (Expedeon, UK) following manufacturer instructions.

Detection of myrosinase activity in SDS-PAGE gel

SDS PAGE gel was prepared as mentioned earlier. The boiling step of sample was omitted. The removal of SDS from the gel was done according to the procedure described by Blank et al., (1982). After electrophoresis the gel was washed (3 Xs, 30 min) with CPB (20 mM, pH 6.0) containing isopropanol (25% v/v) (Blank et al., 1982). After the final wash the gel was incubated (25 °C, 1 h) with either esculin or PNPG.

Results & Discussion

The purpose of this experiment was to develop a simple and rapid method for isolation and purification of bacterial myrosinase using native polyacrylamide gel and esculin for *β*-glucosidase activity assay. The use of esculin for *β*-glucosidase activity was originally described by [14]. In the present study myrosinase activity of *Citrobacter* Wye1 was detected in native PAGE in the sinigrin-induced cells but not in the control without sinigrin (Figure 1). Subsequently the coloured band of active myrosinase was cut and isolated for SDS-PAGE. Analysis of isolated active band of proteins on SDS PAGE (Figure 2) showed few protein bands (5 to 6 bands) suggesting one of the bands was bacterial myrosinase. Analysis of peptide sequence of the band of 66 kDa shows the same results reported by [1], this is a strong evident of using the current method. This method can be used as purification step and therefore allows a shortcut protocol for protein sequencing. The current protocol is comparable with other protein purification methods such as ion exchange & gel filtration used in our lab to purify this protein (data not shown). Such purification methods require large scale (0.5-1 L) media supplemented with highly cost inducer (sinigrin, 10 mM) and high maintenance fees. On the other hand, the current method only 20 mL of culture medium (M9) plus sinigrin (10 mM) is required, thus our method is more cost effective in terms of inducer (sinigrin). Unlike other glucosidases the activity of *Citrobacter's* myrosinase used in this study could not be recovered after removal of

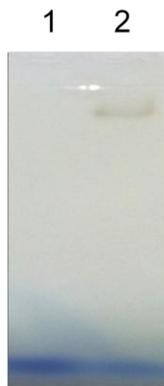
SDS of PAGE, otherwise repeating this method once more may yield a pure and active *Citrobacter* myrosinase. In this case a single gel filtration step may result in pure and active myrosinase protein. Our protocol is faster than the method described by [3] that required sinigrin (0.5 mL) of 12 mM and barium sulphate (0.5 mL) of 18 mM and longer incubation time (30 min or more).

Our method is significantly reduces the amount of sinigrin used and thus represents a huge cost saving to compared with others.

Conclusion

The above method can be used in the following purposes:-

1- As a shortcut to peptide sequencing of bacterial myrosinase
2- Effective a protein purification method especially if followed with traditional methods such as gel filtration chromatography.
3- It allows screening and detection of bacterial myrosinase from several bacterial proteins running on SDS PAGE at the same time.
4- As a way to reduce cost of used glucosinolates (sinigrin) and time for myrosinase isolation.



Native Page assay with esculin reagent: after electrophoresis the gel was incubated with esculin reagent; 1= control (cells was grown on glucose); 2 = sinigrin-induced cells, dark brown band indicated activity of *Citrobacter β*-glucosidase. This experiment was repeated three times.

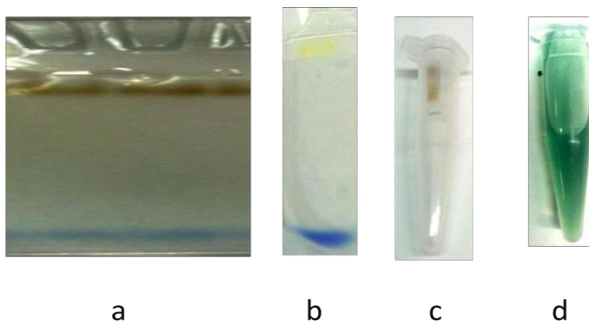


Fig 1 Native gel with esculin reagent from left; (a) the top dark brown colour is indication of esculetin product from esculin hydrolysis by *β*-glucosidase activity; (b) the yellow stain was p-nitrophenol product caused by *p*NPG hydrolysis by *β*-glucosidase; (c) The cut band of the esculin reaction was then assayed for *β*-glucosidase activity by using sinigrin; (d) A positive reaction was observed as green product from GOD-PERID assay suggesting myrosinase activity occurred

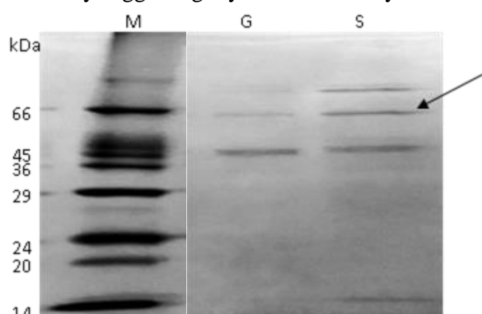


Fig 2 SDS-PAGE of partially purified *β*-glucosidase of *Citrobacter* isolated from native PAGE and run on SDS gel. M = marker; G = extract from cells grown on glucose(non induced cells; S= sinigrin-induced extract from induced cells.The highlighted band represents *Citrobacter* wye1 myrosinase.

We then confirmed the protein band as myrosinase after we conducted peptide sequencing as indicated in our previous research [1]. Thus, we concluded that simple and inexpensive technique using polyacrylamide gel can be used to detect and to isolate myrosinase.

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