Protoplast isolation from leaf mesophyll of sugarbeet *Beta vulgaris* L. axenic seedlings

عزل البروتوبلاست من النسيج المتوسط لأوراق بادرات البنجر السكري المعقمة *Beta vulgaris* L.

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Abstract

Protoplasts were isolated from leaf mesophyll of sugarbeet (*Beta vulgaris* L.) axenic seedlings. Eight enzyme mixtures were tested for cell wall degrading ability. The efficient enzyme solution was mixture "II" that consist of 1.5% Cellulase RS, 2% Cellulase R10, 1% Macerozym R10 and 0.1% Pectolyase Y23. This mixture was efficient in releasing protoplasts and gave high yield of a density 7.3×10^4 protoplast / ml. These isolated protoplasts were viable 93%, their sizes ranged from 13 up to 52 µm, vacuolated and unvacuolated. This finding enable workers to focus on somatic hybridization through protoplast fusion to overcome some barriers facing gene transfer to improve plant species such as transfer of N_2 fixation ability to non-legumes and producing resistant varieties to biotic stress and other traits.

المستخلص

تمكنت الدراسة الحالية من عزل البروتوبلاست من ميزوفيل اوراق بادرات البنجر السكري المعقمة. اختبرت قدرة ثمانية محاليل انزيمية لهضم الجدر الخلوية. واظهرت النتائج ان المحلول الانزيمي "II" الذي يتكون من انزيمات Cellulase RS %1.5، Cellulase R10, %2 Macerozym R10 %1, و Pectolyase Y23 %0.1 تفوق عن بقية المحاليل الانزيمية في سرعة هضمه للجدران الخلوية وتحريره للبروتوبلاست الذي بلغ ناتجه 7.3×10^4 بروتوبلاست / مل. وسجلت حيوية البروتوبلاست المعزول 93% وتراوحت حجوم البروتوبلاست المعزول بين 13 – 52 مايكروميتر بنوعيه الحاوي على الفجوه او الفاقد لها . ان ايجاد بروتوكول كفوء لعزل البروتوبلاست يمثل نقل وادخال تكنولوجيا جديدة معروفة عالميا غير شائعة في بلدنا. وتساعد هذه التقانة الباحثين بالتركيز على عمليات التهجين الجسمي بين النباتات وعلى سبيل المثال نقل صفة تثبيت النتروجين الجوي الى النباتات غير البقولية او انتاج نباتات متحملة لظروف الشد البيئي وصفات اخرى متعددة .

Introduction

Protoplasts isolation provides an excellent experimental material for genetic manipulation of plants through somatic hybridization, cybridization, and transformation procedures [1]. The enzymatic method for protoplasts isolation was first found in 1960 [2], Other investigators isolated protoplasts by mechanical disruption and by enzymatic degradation of their surrounding cell wall [3]. The enzymatic digestion method is now employed routinely for protoplasts isolation. Since it gives numerous amounts of viable protoplasts readily. In general, leaf tissues excised from young seedlings are used extensively as source material for protoplasts isolation [1], because they have been cuticles than other plant parts. Sugarbeet (*Beta vulgaris* L.) protoplasts are considered a recalcitrant material with *in vitro* conditions, particularly their suitability for genetic manipulation within

Key words: Protoplast isolation, leaf mesophyll ,sugarbeet , axenic seedlings

different species of sugarbeet [4]. For isolating protoplasts, sugarbeet greenhouse-grown plants are not only the problem in obtaining sterile isolations but also give a subsequent poor protoplast survival rate [5]. The enzymatic procedures was successful in releasing protoplasts from other tissues such as root hairs of *Lotus corniculatus* [6] root hairs of the forage legume *Trifolium repens* [7] and from agrobacterial transformed hairy roots [8] and from mesophyll to produce somatic hybrids in tobacco [9].

The aim of this investigation was to find out a protocol for isolation of viable protoplasts from mesophyll leaf of sugarbeet.

Materials and methods

Source of plant tissue

Seeds of sugarbeet, var. Baraka were obtained from the General State of Producing Sugar, Mosul, IRAQ, surface sterilized for 10 min. with 6% NaOCl, washed thoroughly with dist. water and germinated in dark at 25±2ºC on agar-solidified MS medium [10]. Leaf mesophyll tissues of the 4-6 wks. Old seedlings were considered as a source material for protoplast isolation.

Enzyme source:

The following enzymes Table (1) were used to prepare different enzyme solutions.

Table (1): Enzymes and their supplier used in this study

Enzyme	Supplier
Cellulase RS	Kinki Yakult manufacturing Co Ltd., Japan
Cellulase"Onozuka" R10	
Cellulysin	Cal-Biochem Behring Corp, USA
Driselase	Freehold, New Jersey, USA
Macerozyme R10	Rhom & Hass, Philadelphia, USA
Pectinase	Seishin Pharmaceutical Co. Ltd. Koamicho Nihonbashi, Tokyo, Japan
Pectolyase Y-23	

Enzyme mixtures preparation:

The seven different enzymes including Cellulase RS, Cellulase R10, Cellulysin, Macerozyme R10, Pectinase, Pectolyase Y-23 and Driselase were used in preparation of various enzyme solutions as mentioned in Table (2).

Table (2): Enzyme solutions used for protoplasts isolation from leaf mesophyll of sugarbeet (*Beta vulgaris* L.) axenic seedlings.

Enzymes		Mixtures							
		I	II	III	IV	V	VI	VII	VIII
Cellulase RS	(%)	1	1.5	0.5	0	1	1	2	2
Cellulase R10	(%)	2	2	0.5	1	0	0	0	0
Cellulysin	(%)	0	0	0	0	0.1	0.5	0.1	0.5
Macerozym R10	(%)	0.5	1	0	0	0	0	0	0
Pectinase	(%)	0	0	0	0	0.1	0.1	0.1	0.1
Pectolyase Y-23	(%)	0.1	0.1	0.1	0.1	0	0	0	0
Driselase	(%)	0	0	0	0	0.1	0	0.1	0
Mannitol	(%)	9	9	4	4	9	9	9	9

All concentrations are w/v in distilled water

These enzyme mixtures were prepared by dissolving the required amount of enzyme powder in 4% or 9% w/v mannitol solution of pH 5.8, and were filter sterilized using Millipore filter of 0.45µm size openings (Chm Lab. group, SPAIN).

Protoplasts isolation

Leaves were excised from 4-6 wks old sterile sugarbeet seedlings, cutting leaf lamina into small (1-2mm^2) pieces, after peeling-off the lower epidermis was incubated in enzyme solutions. One milliliter of enzyme solution was added for each 100 mg leaf tissues in 5.0 cm diam. plastic Petri-dishes [11]. Specimens were kept on horizontal shaker at 30 rpm in dark at room temperature. Protoplasts release was monitored under microscope (BioLab Lines 1007, Taiwan). The enzyme solution containing the released protoplasts was then clarified through a nylon sieve of 45µm openings (PGMG, Nottingham, UK). The enzyme-protoplasts mixture was centrifuged (100g/ 5.0min) and the supernatant removed. Pelleted protoplasts were washed twice by resuspension and centrifugation in 5.0 ml of CPW solution [12] containing 13% w/v mannitol (CPW13M). Subsequently, protoplasts were suspended in KM8P medium [13].

Protoplast characterizations:

1. Size:

Size of the isolated protoplasts was measured using a Leitz-Wetzler microscope with eye piece [14]. As well as, protoplast appearance including shape, membrane integrity and retention of chloroplasts. Volumes were calculated from these measurements, assuming sphericity.

2. Yield:

Protoplasts yield was determined by counting using Fuchs Rosenthal hemacytometer chamber. The usefulness of each enzyme solution was evaluated on the basis of protoplast yield [11]. The working density of protoplasts was adjusted to 1×10^4 protoplast / ml.

3. Viability:

One ml samples of protoplasts suspension were stained with 0.5% (w:v) Evans blue [14] and left for 10 min, then the number of dead protoplasts (blue color) and viable protoplasts (unstained) were counted using a haemacytometer.

4. Nucleation:

Observation of the nuclei in protoplasts was carried out by mixing one volume of the protoplast suspension with 9 volumes of the fixative [15] at 5ºC for 24 hrs., then 50µl of carbol fuchin stain solution was gently mixed with a 20 µl drop of the fixed protoplast [8].

Result and discussion

Assessment of enzyme mixtures

Results indicate that seven out of eight mixtures were sustained the release of protoplasts from leaf mesophyll. The most commonly used source of plant protoplasts is the leaf tissue since it allows the isolation of a large number of uniform cells [16]. Also, because the mesophyll cells are loosely arranged, so that the enzymes have an easy access to the cell wall. These mixtures varied in their effectiveness which influenced maturity and protoplasts yield table (3). Several methods can be followed to facilitate the penetration of enzyme solution into the intracellular space of leaf tissue. The most commonly practiced method is to peel-off the lower epidermis or to cut the leaf tissue into small pieces [17]. We obtained satisfying results when we combined both of removal of the lower epidermis from leaf and then cutting it into 1-2 mm^2 pieces.

Table (3): Effect of enzyme treatment duration on viability and yield of protoplast isolated from leaf mesophyll of sugarbeet (*Beta vulgaris*) axenic seedlings.

Enzyme mixtures	Releasing time (hr.)	Yield (× 10^4 protoplast / ml)	Viability (%)
I	2.00	3.0	77
II	1.00	7.3	93
III	-	-	-
IV	4.00	1.6	73
V	1.30	1.8	92
VI	24.00	1.5	80
VII	2.00	1.3	54
VIII	3.30	1.08	67

Several factors, such as preplasmolysis, osmotic pressure and enzyme composition of the digestion medium are extremely important to obtain highest yields of uniform and healthy protoplasts [18]. The enzyme mixture "II" was found to be the most effective combination in yield 7.3 × 10^4 protoplast / ml^{-1} of viability 93% table (3). The optimal incubation time for protoplast isolation in this solution was one hr. The other enzyme mixtures acted slower, producing somewhat lower protoplasts yield. The optimal time of incubation for these solutions proved to be 1.30-24hrs. The protocol provides a simple and an easy-to-handle procedure that ensured satisfactory yields and quick recovery of

viable protoplasts [19]. Protoplasts were isolated from all treatments, only enzyme mixture “III" proved to be ineffective.

Protoplast size distribution

Protoplasts size was ranged from 13 up to 52 µm distributed as in Figure (1).

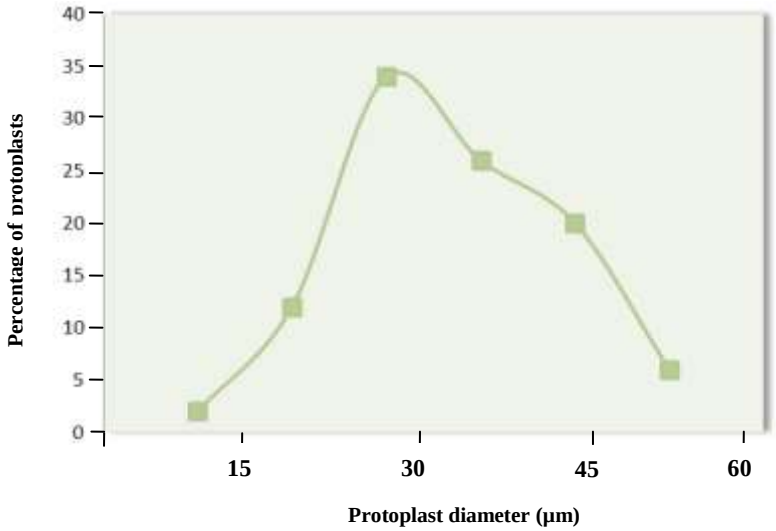

Fig. (1): Size distribution of freshly isolated protoplasts from leaf mesophyll of sugarbeet (*Beta vulgaris*) axenic seedlings

Protoplast shape and chloroplast distribution

Light microscope examination showed that undetached protoplasts were spherical in shape Figure (2,a). Two types of protoplast were identified. One type was unvacuolated Figure (2,b) with regular distribution of chloroplasts, and could be seen to be formed from the cells of the unvacuolated meristematic region. The other type of protoplast was vacuolated Figure (2,c). Prior to their liberation, these vacuolated protoplasts could be seen inside the remains of the partially digested cell walls [2]. Nucleated was 97% Figure (2,d).

In conclusion, the introduction of this protocol in our Lab. with further studies will optimize conditions required for successful protoplasts culture and plant regeneration. This system can be beneficial in genetic manipulation of plants aimed to improve plant species through somatic hybridization and gene manipulation.

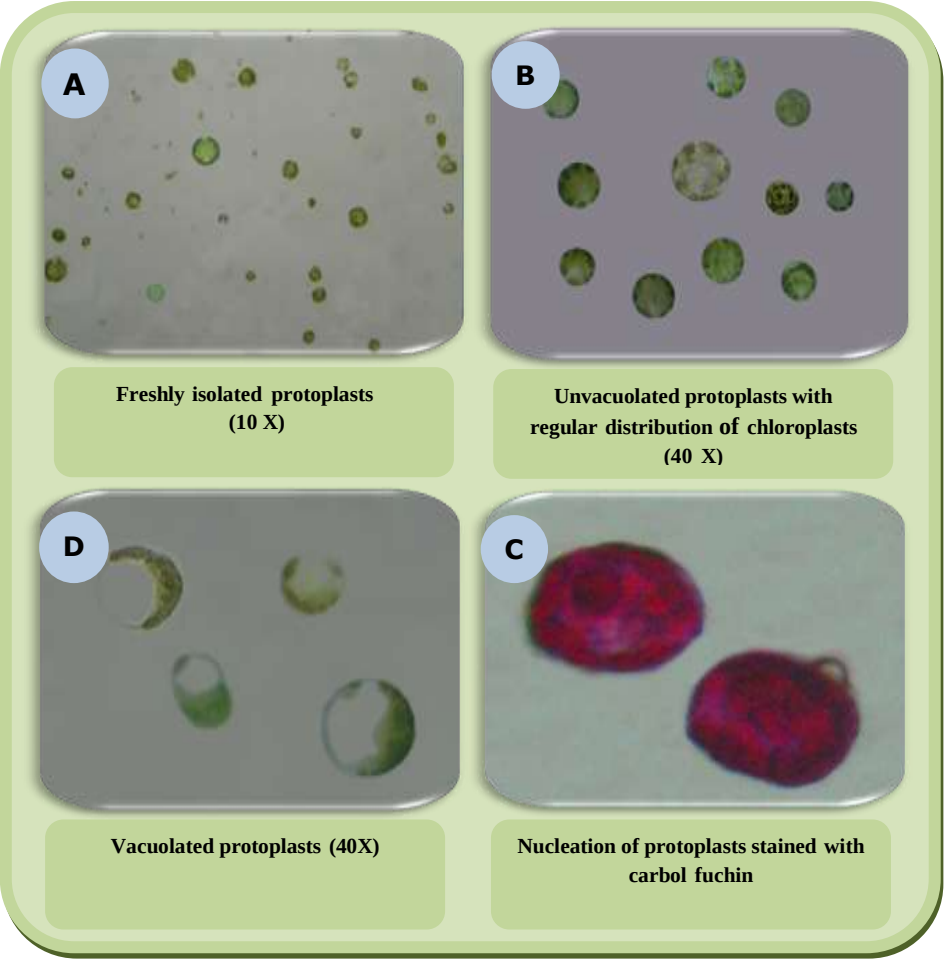

Fig. (2): Isolation of protoplasts from leaf mesophyll of sugarbeet

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