Direct staining for protein bound starch of potato granule- by using Ethidium Bromide dye

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Abstract: First attempt for direct staining of potato starch granule-associated protein (SGBP)by using ethidiun bromide dyes. Light microscopic examination of dye–treated granules showed fluorescence under U.V beam at 230 nm. Colorimetric determination of total proteins associated potato starch granules based on the principle of the Biuret reaction (copper salts in an alkaline medium) and were 0.035 g protein /10 ml (starch =10% g), as well as nitrogen contents (0.0672 g/100g) starch by Kjeldahl. Potato starch granule associated protein revealed as a band ~ 92 kDa in sodium dodecyl sulfate and analysis by gel electrophoresis (SDS page 12% acrylamide).
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1. Introduction

A film of protein wheat starch granules changed from hydrophilic to lipophilic by chlorination or heat treatment (Seuguchi, 1984a, 1984b, 1985, 1986). Protein extracted from starch granules by using salt and tentatively associated with the surface of the granules (Lowy *et al.,* 1981). The starch granule-associated proteins or enveloped starch granule protein known as the proteins found in natural state and its position in and on starch granules (Baldwin, 2001). Storage proteins different from bound tightly on the surface of granule starch to integrated conformation within the granule structure, and these proteins have a MW ~ 5–149 kDa (Israkarn *et al.,* 2007), and known as starch biosynthetic enzymes. Proteins presents in low quantities, the starch associated proteins influence on the rheological granule and rheological characteristics of maize paste (Israkarn *et al.,*2007).

Great importance for better understanding and visualization of starch granules and granule residual by studying the behavior of starch and starch pastes (vande Velde *et al.,* 2002). Compound microscope used for studying the characteristic shape, granule size distribution of starch from different type of plants (van deVelde *et al.,* 2002).As with most fluorescent compounds, ethidium bromide is aromatic. Ethidium bromide is a polycyclic fluorescent dye binds to double strand DNA molecules by intercalating a planar group between the stacked base pairs of the nucleic acid (Waring *et al.,* 1965). When exposed to ultraviolet light, it will fluoresce and appeared with orange colour, intensifying almost 20-fold after binding to DNA.

Little information and studies about using ethidium bromide for direct staining of proteins. So, the aim of this study is to prove that ethidium bromide can be used for the staining of protein enveloped granules of potato starch, detect the granule-associated proteins (protein film enveloped granules) on it and visualization by using compound microscope under U.V beam of 230 nm. Determinations of total proteins of starch granules and its molecular weight by sodium dodecyl sulfate–poly acrylamide gel electrophoresis (Hanand Hamaker, 2002).

2. Materials and methods

Chemicals:

Potato starch granules: normal potato starches obtained from BDH Chemicals Ltd Pool England (Product No. 10271)
Ethidium bromide 1% (10 mg/ml) (Roth, Germany, Art. No. 2218.2).

Compound microscope

Wolfe DigiVu 2.0 Research Digital Microscope (Carolina Biological Supply Company). U.V lamp 230 nm.

Determination of Total Proteins:

Kits for protein determination (bioMerieux, France) at wavelength 545 nm. One gram of protein was grind in grinder and 100 ml sterilized distilled water added, then centrifuged at 5000 rpm for 30 min. The total protein can be calculated from the following equation:
A sample/A standard *n
n= 10: g/100 ml
n=100: g/l
0.25 g of starch equal 0.1 N NaOH (amin determination)

Total Nitrogen Measurement

The method of Kjeldahl used to analytical find the quantitative organic nitrogen level in a liquid (Official Methods of Analysis, 1990)

Gel Electrophoresis of Proteins associated granules of potato Starch

The protein composition of potato starch granules separated on SDS-polyacrylamide gels containing 10% (w/v) acrylamide; (Vos-Scheperkeuter and Witholt, 1984; Wensink and Witholt, 1981). Samples prepared by using dry starch granules (3 mg) suspended in 80 µl denaturation buffer (containing 2% [w/v] SDS and 5% [v/v] (β-mercaptoethanol), (Wensink and Witholt, 1981). The extraction encountered by boiling dry starch prepared previous for 10 min. Samples centrifugation (at 10.000 g for 5 min) and the sediment extracted once more with 40 µl of denaturation buffer then, the supernatants subjected to SDS-gel electrophoresis. Staining of proteins by Coomassie brilliant blue(Vos-Scheperkeuter and Witholt, 1984)

Isolation of Starch Ghosts

According to Hanand Hamaker,(2002) with some modification, 0.5 g of starch suspended in 200 mL of purified distilled water. The suspensions heated to different temperatures at 70 and 100°C on a hot plate stirrer (Fabr. Nr. 659475, Janke & Kunel GmbH & Co. KG), the rate of heated temperature continued for 5–7 min and stir rate 140 rpm. Centrifugation of gelatinized starch at 5000 RPM (Hermle Labor technick, GmbH, SN: 58110042), for 20 min. The upper clear layer of suspension included enveloped protein and the bottom thick rich in starch ghost's phase. The upper and bottom layers examined using light microscope stained by 1% ethidium bromide solution.

3. Results and Discussion

Compound Microscopy

Normal potato starch granules, ghost and gelatinized stained with ethidium bromide (before and after exposed to U.V lamp), examined by compound microscopic showed the proteins exist in intact starch granules, and appeared with light orange colour (Figs 1, A, B, C). It appeared obvious fluoresce with an orange colour when exposed to ultraviolet light (Figs 1, D, E and F). Potato starch granules contained a very thin layer of protein enveloped its surface and by using ethidium bromide, it bind tightened so difficult to remove by washing with water. These results agreed with that showed, the distinct locations of protein starch granule using a specific dye and confocal laser scanning microscopy (clsm), (Han, 2002). Clsm capable to distinguish fluorescence-labeled protein distribution in an optical slice of an intact starch granule. With these techniques, proteins of potato starch granule appeared to be concentrated in internal concentric spheres (Han, 2002). Spheres protein on the surface of potato starch distinguished more than in other types of botanical starch (Han, 2002). Ethidium bromide reacted with amines in proteins and fluorescent granules appeared when UV beam sheds on it. Starch granules heated to 70°C and through optical sectioning of stained granules with ethidium bromide, the compound microscope capable to detect fluorescent- labeled of residual proteins on the granule surface starch (Figs. 2, G and H). Since heating temperature rose to 100°C, the protein accepted staining with ethidium bromide (Fig. 2 I) but not fluoresce when exposed to U.V beam as normal granules or granules which heated to 70 °C, this may be due to changing in the protein construction or shadow behind the thin layer of enveloped protein for amylase and amylopectin disappeared. Three processes encountered during gelatinization of starch granules: swelling, crystal or double helical melting and amylose leaching(Jenkins and Donald,1998).

Proteins isolated from gelatinized and ghosts starch

The heated potato starch granules to 100°C, separate the protein which tightened with it. The polymers of starch may be not associated with soluble proteins but when washed in enough warm water of 40°C, lead to leach out of it. On the other hand, the high tightened of gelitanized starch granule suggests a possible role in maintaining proteins in the starch granular construction of ghosts and effect on viscosity and pastes breakdown (Miller *et al.,* 1973; Evan and Haisman, 1997). The starch gelatinization loss crystalline state of the granules by increasing starch solubility in high alkalinity and this due repulsion of charges between chains (Evan and Haisman, 1997), and caused breakdown of hydrogen bonds into molecular in random coil structure of amylose, starch granules start to degradation (Han and Lim, 2004). Result in Table 1. showed the total protein on potato starch granules, which determined by Biuret reaction (copper salts in an alkaline medium 0.035 g / 10 ml of starch solution 1%, as well as nitrogen contents by Kjeldahl were 0.0672 g/100 g starch.

Proteins extracted from potato starch granules

The extracted proteins subjected to SDS-poly acrylamide gel and the resulting proteins bands compared with low molecular weight protein marker (Amersham); Fig. 2; showed the profile of protein tightened to starch granules. One major protein was found and higher in its molecular weight ~92 kD**.** Protein of the starch granules known to be appeared in different structures and its molecular weight is high (Baldwin, 2001). The starch granules tightened protein have been declared that the MW of ~ 65, 68, 70, 72, 74–79.9, 83, 90–93, 100, 105 and 140 kD and status contingent on its source (Baldwin, 2001).

Isoform terminology in maize starch granule tightened protein molecular weight ~ 93 kD, was isolated and termed SGBSS II (Preiss, 1988). Two SGBSS with molecular weights of ~ 59 and 77 kD, were isolated from starch of pea and called GBSS I and GBSS II, respectively, (Smith, 1990; Dry*et al.,* 1992; Denyer, 1993; Baldwin, 2001). Protein with molecular weight ~83 kD SGBSS found in maize, ~ 77 and 90 kD found in barley, and protein of ~ 92 kD SGBSS II isolated from potato starch granules (Edwards *et al.,* 1985). The primary amino acid sequences of the 77 kD for SGBSS II in pea and wheat starch and has 92 kD for SGBSS II in potato starch protein (Edwards*et al.,*1985; Smith *et al.,* 1990; Denyer *et al.,* 1993). Protein with molecular weight ~60 kD appeared to be returns to isoform I (Baldwin, 2001), (independently of botanical source), the expression isoform II can returns to proteins itscharacterized different in molecular weights (Hylton *et al.,* 1996).

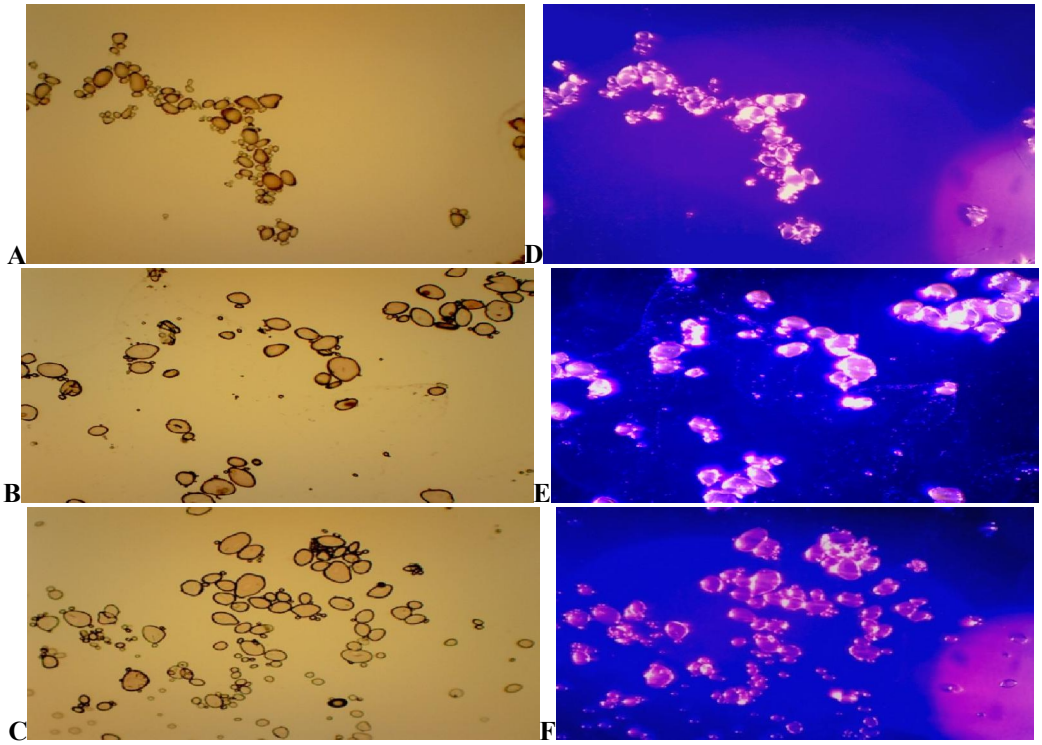

Figure 1. Pictures of starch granules stained with ethidium bromide under compound microscope (A, B and C) and after U.V beam sheds (D,E and F).

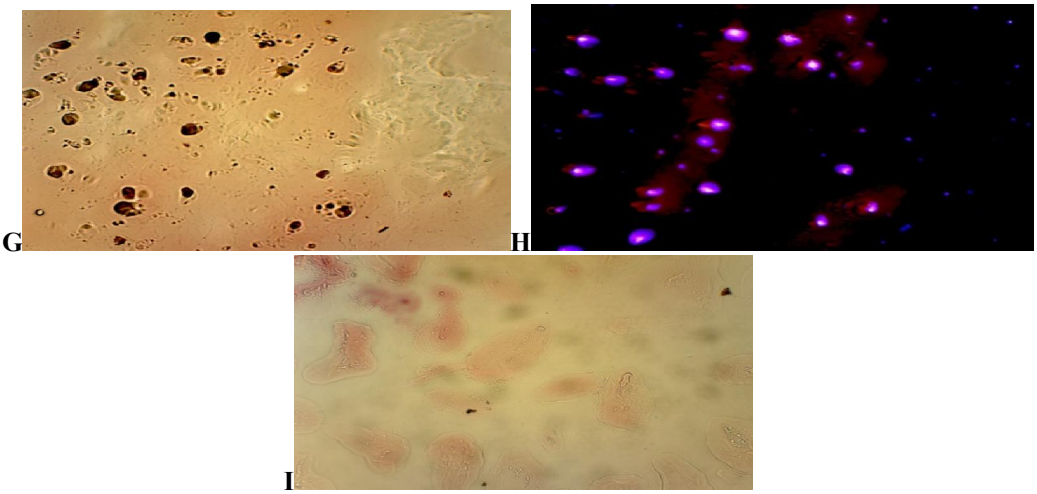

Figure 2. Picture of G and H: a partial gelatinized starch granules befor and after U.Vsheds, I: complete gelatinized starch granules stained with ethidium bromide appeared only by normal light of microscope and disappeared after U.V beam used.

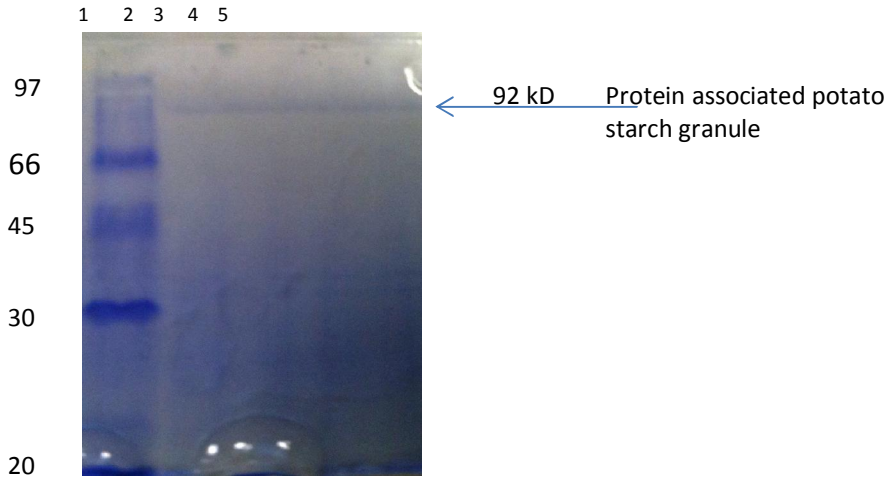

Figure 3. lane 1: low molecular weight protein marker (Amersham), lane 2, 3, 4 and 5: extracted protein associated potato granules.

Table 1.Determination of total protein associated potato starch granules

Sample	Absorbance (545 nm)
Blank	0.000
Bovine serum albumin	0.084
Starch	0.003
Total protein= (0.003-0.00/0.084)* 1 = 0.035 g/10ml	

4. Conclusions

Staining of potato starch granule by using ethidium bromide and exposing to U.V lamp, the granules appeared orange under the compound microscope proof, that the granules surrounded by a film of protein. The molecular weight of the protein associated potato starch on SDS page was ~92 kD.

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