Original Article

Substitution of cassava starch for polyvinyl alcohol in the histochemical stain for glucose-6-phosphate dehydrogenase in animal tissues

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Abstract

The histochemical localisation of glucose-6-phosphate dehydrogenase (G-6-PD) in tissues, using aqueous media is problematic because more than 90% of the activity of this enzyme is lost in the media, thereby giving a value that is much less than the real value. The gel method was tried to solve this problem but with little success. The improved method by Negi and Stephens, which incorporated 22% polyvinyl alcohol (PVA) in the incubation media, was an improvement in the histochemical demonstration of G-6-PD activity in tissues. In this investigation, we used 5% cassava starch, instead of PVA. Result showed improved localisation of the activity of G-6-PD in the liver and testis of Sprague Dawley rats which was statistically better than the Negi and Stephens' method. We conclude that cassava starch is a better, safer and cheaper substitute to PVA, in the localisation of the activity of G-6-PD in animal tissue.

Key words: Cassava starch, polyvinyl alcohol, cyanogenic glycosides, glucose-6-phosphate dehydrogenase

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G-6-PD) is the first enzyme to catalyse the rate-limiting irreversible oxidation of glucose-6-phosphate in the pentose phosphate shunt. It is widely distributed in animal and plant tissues (Negi and Stephens, 1977).

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The activity of this enzyme is high in blood cells, adipose tissue, steroid–producing cells and lactating mammary glands, but low in liver cells (Pearse, 1988).

Histochemical localisation of G-6-PD in aqueous media results in loss of more than 90% of the cytoplasmic dehydrogenase content of the enzyme from the cytoplasm. This loss is reduced considerably with the incorporation of 22% of PVA, an inert tissue stabiliser in the incubating medium (Negi 1977; Frederiks *et al.*, 2006).

Tetrazolium salts were introduced to demonstrate dehydrogenase activities in tissue sections with the formation of a coloured precipitate, formazan about 45 years ago (Buthcher and Chayen 1965; Gahan and Kalina, 1968; Altman, 1969). Nitro Blue Tetrazolium (NBT) was one of the best tetrazolium salts used for quantitative purposes (Henderson and Loveridge 1981; Kugler 1982; Van Noorden and Butcher, 1984). The tetrazolium method which works on the reduction of tetrazolium salt to produce formazan is the most widely used method in enzyme histochemistry. This reaction is

illustrated below. However the presence of oxygen in the substrate medium decreases the rate at which formazan is formed. This affects the activity of the enzyme. This interference could be suppressed completely by including azide in the incubation medium (Pearse, 1988). In order to localise dehydrogenases correctly, azide should be routinely included in the incubation medium to a final concentration of at least 5 mM, whenever NitroBT is used (Stoward *et al.*, 1991). Potassium cyanide is often substituted for azide (Van Noorden and Tan, 1982).

Cassava Starch

Cassava starch contains cyanogenic glycosides and free cyanide (Montagnac *et al.*, 2009). Only a negligible amount of cyanogenic compounds remain in starch products. It is estimated that cassava starch products have less than 2 mg of HCN equivalent per kg of starch (Kuakun *et al.*, 2005). It is also reported that starch contains less than 4% of the cyanide, present in cassava (Arguedas and Cooke, 2007). This makes cassava starch, an ideal inert tissue stabiliser, because it contains cyanide, and there will be no need to add azide or cyanide into the medium.

The average density of starch was found to be 6% (Brabander), while the capillary flow method showed a viscosity of 680 (Uhumwango *et al.*, 2005, Endale *et al.*, 2009). This viscosity varied from 7.25 to 13.1 RVU (Magali *et al.*, 2009), while the peak viscosity was reported to be 253.01-344.96 RVU (Nuwamaya *et al.*, 2010).

Cassava starch is produced and used locally in textile industry. It is a non-toxic biodegradable natural product.

The aim of this investigation is to determine whether cassava starch substituted for PVA will improve the histological localisation of the activity of G-6-PD in tissues.

MATERIALS AND METHODS

Preparation of Cassava Starch

Native cassava starch extraction was carried out by peeling 100 g of fresh tubers. This was homogenised with 100 ml of distilled water using a Waring blender. The mixture was stirred with stirring rod for 2 minutes and filtered using a triple cheese cloth. The sediment was allowed to stand for 1 hour to facilitate starch sedimentation, and the top liquid was decanted and discarded. 200 ml of distilled water was added, followed by centrifugation at 3000 *g* for 10 minutes. The starch was air dried on aluminium pans at room temperature for 24 hours.

Tissue Samples

Ten male Sprague Dawley rats weighing more than 350 mg were selected from the Animal House Holding of the Faculty of Medical Sciences, University of the West Indies. The rats were housed in two cages for two weeks to acclimatise. They were given normal rat feed and water *ad libitum* during the time of acclimatisation.

After acclimatisation, the rats were sacrificed following diethyl ether anaesthesia. Liver and testes tissues were obtained from the sacrificed animals. These were quickly frozen. Cryostat sections 10 μm thick were cut from the tissues.

Staining

Two incubation media were prepared; one using 22% PVA as the tissue stabiliser (Stephen and Negi) and another using 5% cassava starch as the tissue stabiliser.

- Incubation media according to Stephen and Negi.

The PVA was prepared by weighing 11 g of PVA into a 125 ml flask containing 50 ml of Tris-maleate buffer, pH 7.2. The flask was covered with aluminium foil, and immersed in simmering hot water to dissolve the PVA. After the PVA has dissolved, the flask was removed from the boiling water and the solution was allowed to cool to room temperature before use.

The incubation medium contained:

- 20 ml of polyvinyl alcohol (22%) in Tris-maleate buffer (0.2 M, pH 7.2)
- NitroBT 10 mg
- NADP 10 mg
- G-6-P 60 mg.

The medium was thoroughly mixed.

- Incubation medium using cassava starch.

Cassava starch solution (5%) was prepared by weighing 0.5 g of dried cassava starch and placing it in a 125 ml flask containing 50 ml of Tris-maleate buffer, pH 7.2. The flask was placed in a beaker of hot water. This was swirled constantly until a viscous solution was obtained. The solution was allowed to cool to room temperature before being used.

The incubation medium contained:

- 20 ml of cassava starch (5%) in Tris-maleate buffer (pH 7.2)
- NitroBT 10 mg
- NADP 10 mg
- G-6-D 60 mg.

Cryostat sections, 10 μm thick, were cut from the frozen liver and testes tissues from each of the 10 rats. Two sections were obtained from each rat for each tissue (liver and testis). One group was incubated in the PVA containing medium while the other group was incubated in the cassava starch containing medium, for 30 minutes

at 37°C, using incubation wells. After the incubation, the slides were washed with distilled water to remove excess medium. The slides were then placed in coupling jars containing distilled water for 1 day to further remove the incubating media. The slides were then left to dry in air before being mounted with Protex.

The areas with G-6-PD activity stained blue.

Quantification Of Enzyme Activity

Ten slides each (PVA and cassava starch) from the liver and the testis were quantified using Image J software (NIH). The histogram of the 8 pixel grey image of 10 slides each (PVA and cassava starch) for the liver and the testis were plotted and the mean staining intensity of each slide was recorded. Image J operates from 0 to 255. Zero represents maximum intensity while 255 represents no stain. Thus lower the mean pixel value of the histogram the greater the staining intensity. With a confidence interval of 95% a *P* value of 0.05 was used to determine the statistical significance between the two techniques.

A representative micrograph of each stain, their 8 pixel grey image and their histograms were recorded for PVA (liver and testis) and cassava starch (liver and testis).

One-way ANOVA statistical software was used to determine the significance of the staining intensities of the two methods.

RESULTS

Results show that the staining intensity of cassava starch is more than that of PVA in the histochemical demonstration of G-6-PD in the liver of Sprague Dawley rats [Table 1, Figures 1 and 3]. The result also showed a similar result in the testis of Sprague Dawley rats [Table 1, Figures 2 and 4].

DISCUSSION

In this investigation it has been demonstrated that cassava starch showed greater staining intensity than PVA [Table 1, Figures 2-4]. This demonstrates that cassava starch is a better substitute than PVA as an inert tissue stabiliser in the histochemical demonstration of G-6-PD in the liver and testis of Sprague Dawley rats.

The mean pixel value for cassava starch was shown to be 98.77 as against 138.99 for PVA in the histological demonstration of G-6-PD in the liver. The *P* value for the staining intensity between the two methods is 0.029, indicating a statistical difference ($P < 0.05$).

In the testis the mean pixel intensity for cassava was shown to be 172.38 while that of PVA is 186.08. The *P* value is shown to be 0.026, which is statistically significant.

Cassava starch is a natural product which is used routinely in the clothing industry. It is degradable and non-toxic. Its low content of cyanide makes it ideal for enzyme histochemistry of G-6-PD, because the cyanide content takes care of the oxygen in the media which interferes with formazan formation. It is cheap and easy to prepare in the laboratory.

We conclude that cassava starch is a better substitute for PVA in the enzyme histochemistry of G-6-PD and other dehydrogenases that are soluble in aqueous media.

Table 1: Mean pixel values for PVA and cassava starch in the liver and the testis

Rat number	Liver PVA	Liver starch	Testis PVA	Testis starch
1	103.88	92.27	189.42	188.23
2	86.79	74024	190.45	188.74
3	160.27	98.75	174.52	171.87
4	161.46	74.81	177.23	173.62
5	182.96	67.53	198.54	163.15
6	183.98	85.88	202.36	162.59
7	103.98	167.43	198.54	165.87
8	85.79	159.12	202.36	165.72
9	178.23	93.09	163.72	170.37
10	142.52	74.59	163.64	173.62
MEAN	138.99±40.11	98.77±16.13	186.08±15.23	172.38±9.39

P value for liver=0.029; testis=0.02, PVA - Polyvinyl alcohol

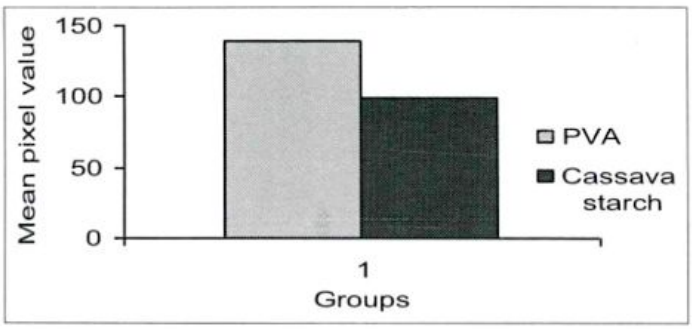

Figure 1: Mean pixel values for PVA and cassava starch in the liver

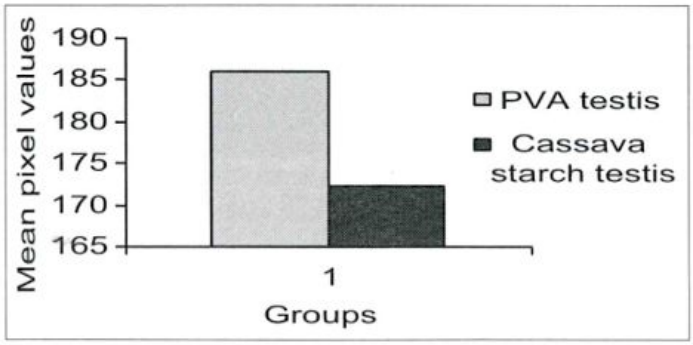

Figure 2: Mean pixel values for PVA and cassava starch in the testis

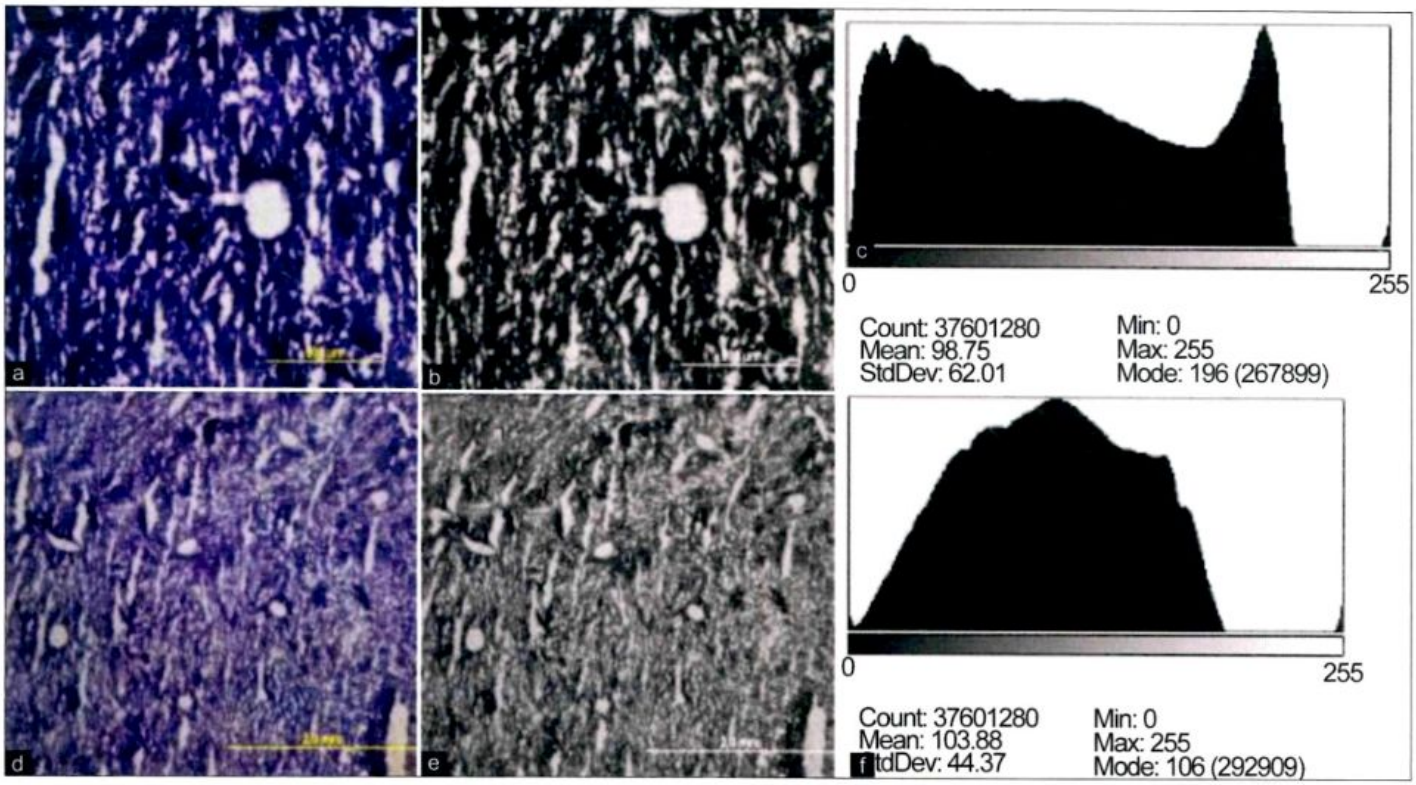

Figure 3: Photomicrographs of the liver stained for G-6-PD activity. (a) Photomicrograph of stain using 5% cassava starch, (b) 8 pixel grey image of (a), (c) Histogram of (b), (d) Photomicrograph of stain using PVA, (e) 8 pixel grey image of (d), (f) Histogram of (e)

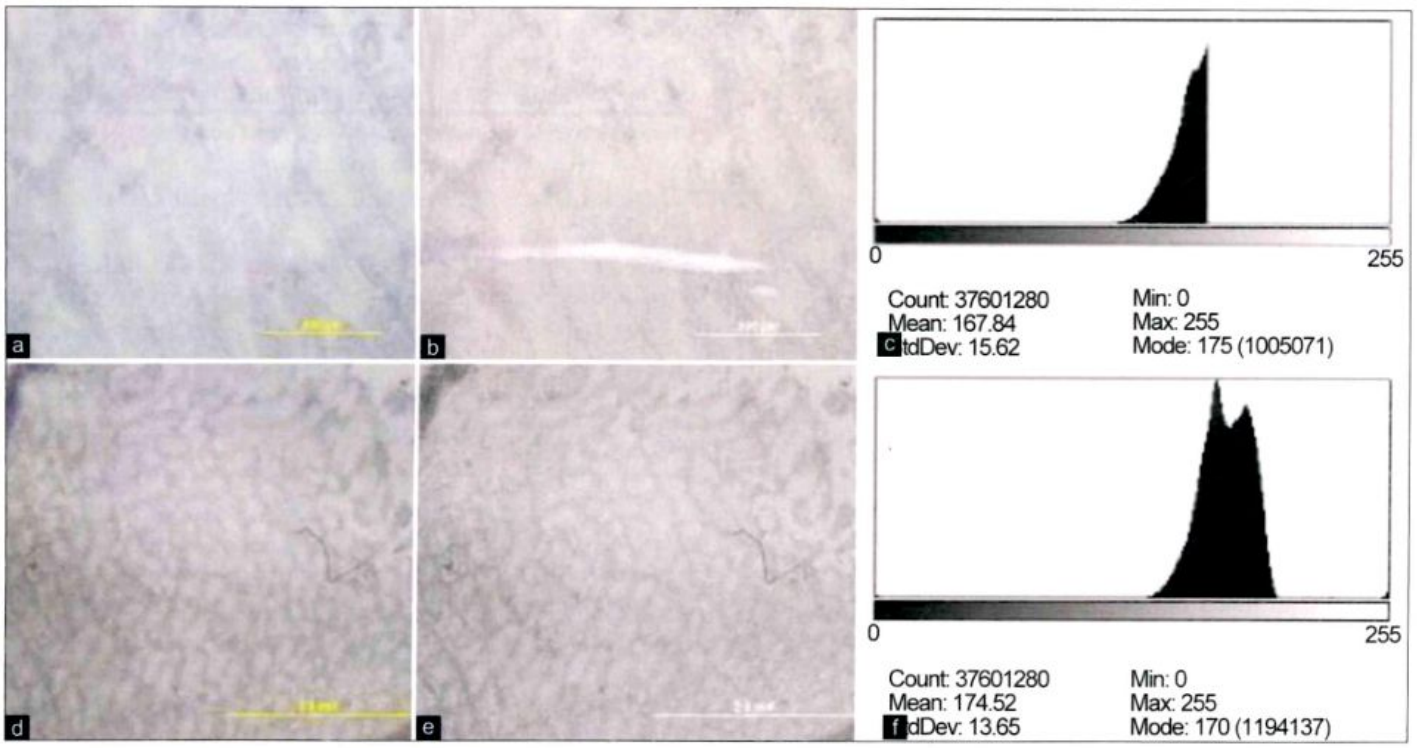

Figure 4: Photomicrographs of the testis stained for G-6-PD activity. (a)Photomicrograph of stain using 5% cassava starch, (b) 8 pixel grey image of (a), (c) Histogram of (b), (d) Photomicrograph of stain using PVA, (e) 8 pixel grey image of (d), (f) Histogram of (e)

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