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Purification and characterization of glycerol phosphate dehydrogenase produced from wheat bran using a mixed culture system

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Glycerol phosphate dehydrogenase was produced from wheat bran by the mixed culture fermentation technique. The process involved saccharification of cellulose to glucose by *Trichoderma viride* followed by conversion of glucose into glycerol by *Saccharomyces cerevisiae* Y-1347. Different purification steps were applied to the culture filtrate to obtain a pure enzyme preparation. The pure preparation indicated that the molecule consists of one peptide chain, with a molecular weight of 51 000 and isoelectric point of 5·7. The amino acid content was also studied. Lineweaver–Burk analysis gave a k_m value of 0·033 mmol and V_{max} of 83·3 mmol/ml/mg protein/min. The enzyme showed its maximum activity at pH 6·0 when incubated at 25°C for 10 min.

INTRODUCTION

One of the most interesting recent approaches in the field of industrial fermentation is the use of mixed microbial culture techniques to produce economic compounds in one-batch fermentations. This technique has been used for production of ethanol from cellulosic wastes in a single step (Freer & Wing, 1985; Dengbo *et al.*, 1987; Daniel *et al.*, 1990). Glycerol phosphate dehydrogenase catalyses a number of metabolic functions, especially conversion of glucose into glycerol (Ostro & Fondy, 1977; Edgar & Bell, 1978; Nilson & Adler, 1990).

No previous studies of glycerol phosphate dehydrogenase production using the mixed culture system with *Saccharomyces cerevisiae* Y-1347 have been carried out. Accordingly, the present work aimed to produce glycerol phosphate dehydrogenase from wheat bran by adapting a mixed microbial culture technique in which two successive transformation process took place. The first step involved the saccharification of cellulosic materials to glucose by the fungus *Trichoderma viride*. This was followed by conversion of glucose into glycerol by the yeast *Saccharomyces cerevisiae* Y-1347. Also, purification and characterization of the enzyme were attempted.

MATERIALS AND METHODS

Microorganisms

Pure stock cultures of the yeast strain a *Saccharomyces cerevisiae* Y-1347 and a cellulose-decomposing fungal strain of *Trichoderma viride* were kindly provided from the Microbiological Resource Centre, Ain Shams University (MIRCEN), Cairo, Egypt. They were maintained on malt extract agar slants and fresh transfers were prepared at monthly intervals. Yeast and fungal inocula were prepared by mixing two slants of yeast or fungus grown on malt agar slant (at 30°C for 48 h and 4 days, respectively) with 10 ml of sterile distilled water. Aliquots of 3 ml of each suspension were used as standard inocula.

The mode of culture and fermentation medium

The bioconversion steps were carried out using the mixed culture system of *T. viride* which converts cellulose into glucose and *S. cerevisiae* Y-1347 for the conversion of glucose into glycerol. The saccharification medium had the following composition (g/litre): yeast extract, 10; peptone, 5; KH_2PO_4, 3; $(NH_4)_2SO_4$, 3; $MgSO_4.7H_2O$, 3; $CaCl_2$, 1; and distilled water to make 1 litre. Wheat bran (4 g/100 ml) was added as the sole carbon source in the first step. At the end of the saccharification period (4 days), the yeast suspension was inoculated to the saccharification mixture. At the same time the following ingredients were added (g/litre): NaCl, 7; $NaHSO_3$, 30; beef extract, 1; Tween-80 (20 mg/ml) and the incubation was continued for a further 48 h.

Determination of glycerol phosphate dehydrogenase activity

This was carried out according to the method of White & Kaplan (1969). One enzyme unit was defined as the

amount of enzyme that catalyses the reduction of 1 mmol dihydroxy-acetone per min.

Purification of glycerol phosphate dehydrogenase

Solid $(NH_4)_2SO_4$ (260 g/litre) was slowly added to the clear culture filtrate. The mixture was left for 2 h at 4°C then centrifuged at 8000 rpm for 20 min at 4°C. The obtained precipitate showed no enzyme activity; therefore it was discarded. The clear supernatant was enriched with $(NH_4)_2SO_4$ (170 g/litre) and, after standing for 1 h at 4°C, the precipitate which contained the enzyme activity was collected by centrifugation under cooling (8000 rpm for 40 min). This precipitate was dissolved in 5mM sodium phosphate buffer (pH 7) and dialysed overnight against the same buffer. The dialysed ammonium sulphate fraction (20 ml) was applied to a pre-equilibrated DEAE—Sephadex A50 column (2·7 × 50 cm). The proteins were eluted with 500 ml of 50mM sodium phosphate buffer (pH 7) with a linear gradient of NaCl (0·01–0·05 M) at a flow rate of 1 ml/min. Fractions (5 ml) were collected using a fraction collector (LKB Ultorace) at 4°C. The fractions were monitored for proteins at 280 nm as well as for enzyme activity.

The DEAE—Sephadex pooled fractions (40–65) were dialysed against 50mM phosphate buffer (pH 6·2), concentrated (5 ml) and applied to a CM–Sephadex C50 column (at 4°C). Elution of proteins was done with 500 ml of 50mM sodium phosphate buffer (pH 6·2) with linear gradient of NaCl (0·01–0·05M) at a flow rate of 1 ml/min.

The fractions were monitored for protein content at 280 nm and also for the enzyme activity. Fractions 35–55 were pooled, dialysed against 20mM sodium phosphate buffer (pH 7·5), concentrated (2 ml) and lyophilised.

Polyacrylamide gel electrophoresis (PAGE) was performed using MONO-PHOR (Labor Müller, Germany), and according to the method of Stegemann *et al.* (1987). SDS–PAGE was carried out in 3-mm slab gels of 6% acrylamide in Tris–borate buffer (pH 7·1) containing 0·1 SDS. Polyacrylamide gel isoelectric focusing (PAGIF) was performed in gel tubes of 6% acrylamide and 1 ampholyte (servalyte T. pH 4–9). Gels were stained with 0·025 Coomassie Blue in a staining solution as given by Stegemann *et al.* (1987).

The amino acid content of the purified enzyme was carried out using a Beckman Amino Acid Analyser (Model 119 GL) according to the method of (Speckman *et al.*, 1958).

RESULTS AND DISCUSSION

Partial purification of the enzyme

Ammonium sulphate (26 and 43 (w/v)) was added to the crude extract of glycerol phosphate dehydrogenase from *S. cerevisiae* Y-1347. It was found that most of the enzyme activity was concentrated in the second precipitate. The specific activity of the enzyme was increased from 2·23 (in the crude extract) to 93·6 mmol/mg protein/min. On the other hand, the total protein was decreased by 98·2% with activity preservation of 75·2% and purification factor of 42·0 fold (Table 1).

The ammonium sulphate fraction was applied to the pre-equilibrated DEAE—Sephadex A5O anion-exchange column. Results in Fig. 1 indicate that, although this fraction contained different protein molecules, only one band showed activity for glycerol phosphate dehydrogenase. Results in Table 1 also show that the enzyme was enriched after the DEAE-Sephadex A50 since the specific activity became 469·0 mmol/mg protein/min with a purification factor of 210.

The enzyme-rich fractions of the DEAE–Sephadex column were dialysed, concentrated and applied to a pre-equilibrated CM–Sephadex C50 column. Figure 2 shows a sharp distinctive band of the enzyme activity which fits completely with one of the protein bands. The specific activity of the enzyme was further increased and become 727 mmol/mg protein/min. The enzyme was purified 326-fold with a yield of 44·4.

Table 1. Purification profile of glycerol phosphate dehydrogenase from *S. cerevisiae* Y-1347

Purification steps	Volume (ml)	Activity (mmol/ml/min) (U/ml)	Protein (mg/ml)	Specific activity[a] (mmol/mg) protein/min)	Purification factor[b] (-fold)	Yield[c] (%)
Culture filtrate crude extract	400	45	20·13	2·23	0·00	100
Precipitate by ammonium sulphate	20	677	7·23	93·6	42·0	75·2
Filtration on DEAE–Sephadex A50	5	1965	4·20	468	210	54·5
CM–Sephadex C50	2	4000	5·50	727	326	44·4

[a] Specific activity = Total activity/total protein.

[b] Purification factor = $\frac{\text{specific activity of purified enzyme}}{\text{specific activity of the crude enzyme}}$.

[c] Yield = total activity of the purified enzyme/total activity of the crude enzyme.

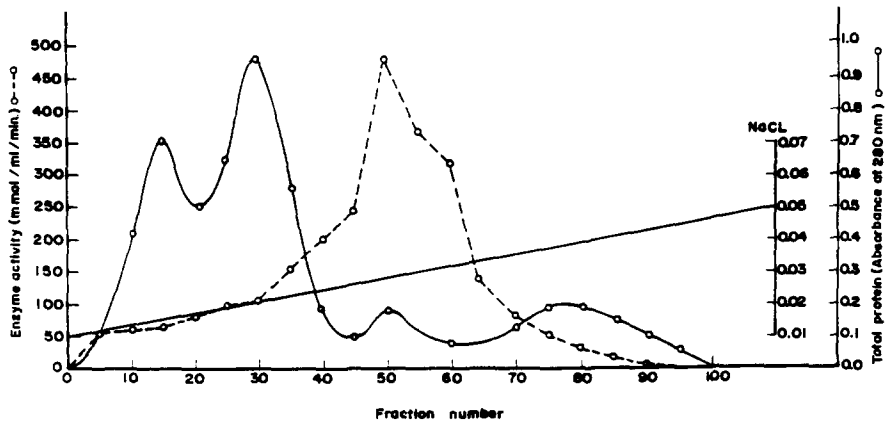

Fig. 1. First DEAE—Sephadex A50 chromatography of glycerol phosphate dehydrogenase. The dialysed ammonium sulphate fraction was chromatographed on DEAE–Sephadex A50 column by elution with a linear gradient of 0·01–0·05M NaCl. Total protein was monitored at 280 nm. The fractions were assayed for enzyme activity.

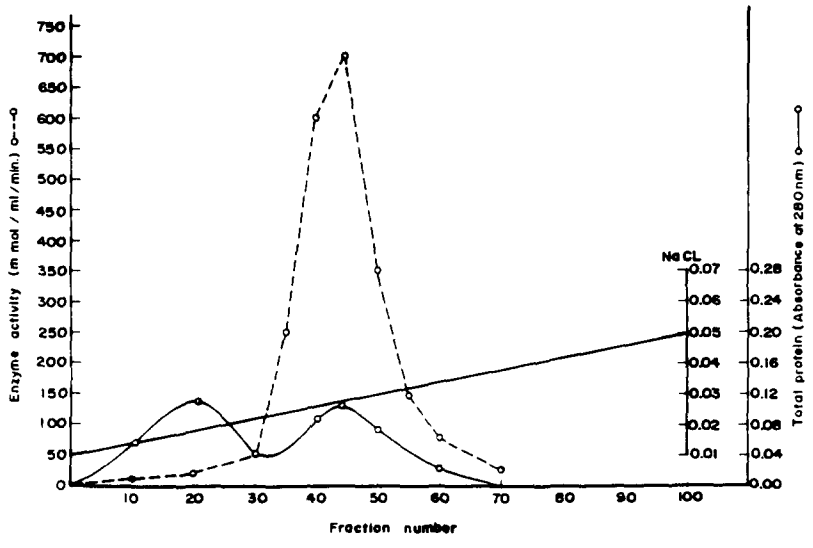

Fig. 2. CM-Sephadex C50 chromatography of glycerol phosphate dehydrogenase. The first DEAE–Sephadex fraction was applied to CM–Sephadex C50. Proteins were eluted with a linear gradient of 0·01–0·05M NaCl. Total protein was monitored at 280 nm. The fractions were assayed for enzyme activity.

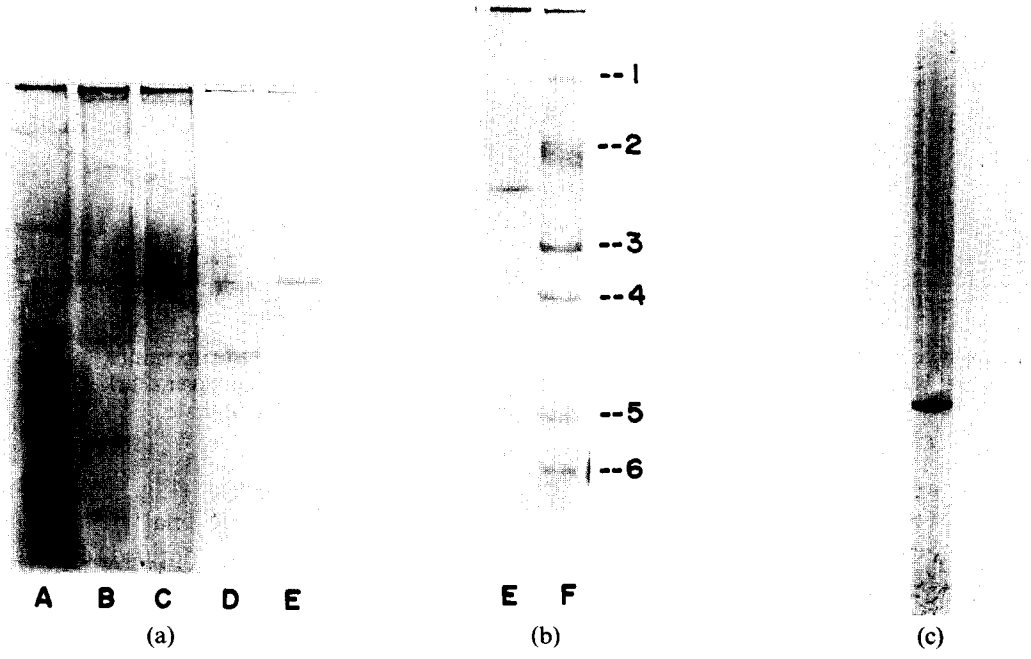

Fig. 3. Electrophoretic properties of glycerol phosphate dehydrogenase from Saccharomyces cerevisiae Y-1347. (a) Disc. PAGE: A, crude extract; B, ammonium sulphate fraction before dialysis; C, ammonium sulphate fraction after dialysis; D, DEAE–Sephadex fraction; E, CM–Sephadex fraction. (b) SDS–PAGE: 1, phosphorylase, mol. wt 97 000; 2, bovine serum albumin, mol. wt 67 000; 3, ovalbumin, mol. wt 42 000; 4, carbonic anhydrase, mol. wt 31 000; 5, soybean trypsin inhibitor, mol. wt 21 500; 6, lysozyme, mol. wt 14 000; F, marker protein. (c) PAGIF.

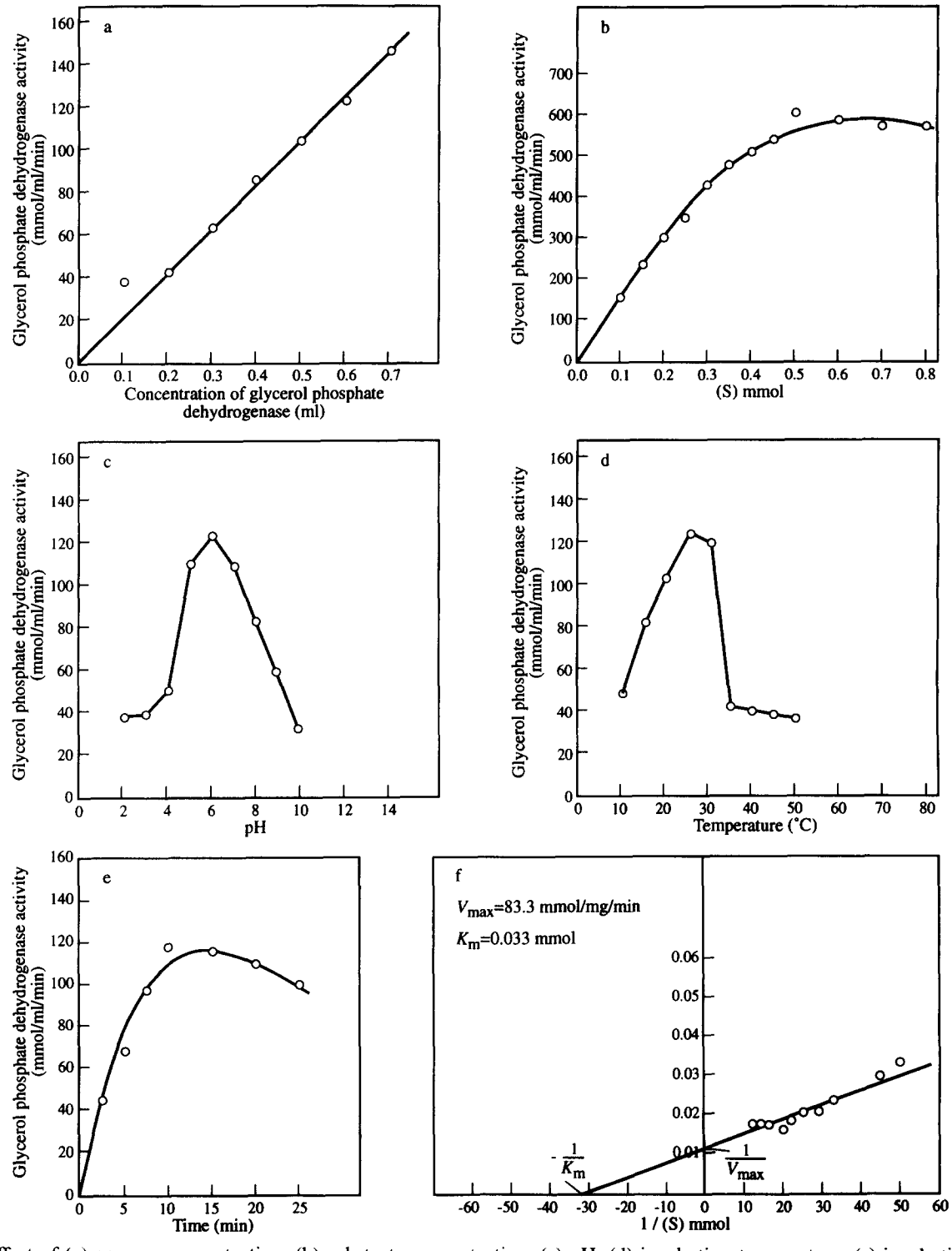

Fig. 4. Effect of (a) enzyme concentration, (b) substrate concentration, (c) pH, (d) incubation temperature, (e) incubation period on the activity of *S. cerevisiae* Y-1347 glycerol phosphate dehydrogenase activity. (f) Lineweaver–Burk plot of *S. cerevisiae* Y-1347 glycerol dehydrogenase activity versus dihydroxy acetone phosphate.

The crude extract, as well as the purification steps of *S. cerevisiae* Y-1347, were monitored using different electrophoretic techniques to study their homogeneity, examine the purity of chromatographic steps and for determination of the molecular weight and isoelectric point the purified enzyme. On using disc. PAGE, the resolved electrophoretic bands were progressively improved from the crude extract to the final step of CM—Sephadex C50 which revealed only one distinctive band, indicating a pure preparation of glycerol phosphate dehydrogenase (Fig. 3(a)). These results were confirmed when the final step was analysed by SDS–PAGE. The finally purified enzyme showed a single band with a relative electrophoretic mobility of 0·39. On comparing this value with those of marker proteins of known molecular weight (Fig. 3(b)), the corresponding molecular weight of the purified enzyme was found to be 51 000.

Wide variation in the molecular weight values of glycerol phosphate dehydrogenases from different

Table 2. Amino acid content (mol %) of the purified *S. cerevisiae* Y-1347 glycerol phosphate dehydrogenase

Amino acids	*S. cerevisiae* Y-1347 glycerol phosphate dehydrogenase
Threonine	3·02
Valine	2·07
Methionine	0·43
Isoleucine	1·52
Leucine	4·60
Phenylalanine	1·35
Lysine	2·21
Aspartic	4·05
Serine	6·07
Glutamic	1·26
Proline	1·47
Glycine	11·1
Alanine	2·91
Tyrosine	0·61
Histidine	6·23
Arginine	0·91

microorganisms was found in the literature. It was reported that the molecular weight for *E. coli* was 32 500 (Edgar & Bell, 1978), whereas for *Candida utilis* it was 71 000 (Halsey, 1982). Schryvers & Weiner (1981) found that the purified enzyme of *E. coli* strain 8 had a molecular weight of 58 000. On focusing the purified fraction in a gel tube it showed one sharp band (Fig. (3)), indicating a highly purified enzyme with an isoelectric point of 5·7. In accordance with the present results, the Edgar & Bell (1978) glycerol phosphate dehydrogenase produced by *E. coli* strain 8 has an isoelectric point equal to 6. Also, Halsey (1982) reported an isoelectric point of 5·2 for *Candida utilis* glycerol phosphate dehydrogenase.

Table 2 presents the amino acid contents of the purified *S. cerevisiae* Y-1347 glycerol phosphate dehydrogenase. The enzyme contained large amounts of leucine, aspartic acid and threonine and proved to be rich in glycine and histidine.

The enzyme activity showed a linear relationship with enzyme concentration (Fig. 4(a)). The relationship between substrate concentration and enzyme activity is shown in Fig. 4(b). The maximum enzyme activity occurred when incubated with optimum substrate concentration at pH 6 (Fig. 4(c)) and at 25°C (Fig. 4(d)) for 10 min (Fig. 4(e)). A Lineweaver—Burk plot (Fig. 4(f)) gives a K_m value of 0·033 mmol and a V_{max} value of 83·3 mmol/mg protein/min.

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