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Isolation and separation of β-galactosidase from *Aspergillus nidulans*

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Abstract

Resolution of β-galactosidase of *Aspergillus nidulans* on DEAE-cellulose revealed two peaks of enzyme activity—designated as Enz A (the 0·2 M NaCl eluted fraction) and Enz B (the 0·3 M NaCl eluted fraction). Enz A was obtained in larger yields and possessed higher activity than Enz B. Purification achieved with Enz A was 10·73 fold while that of Enz B was 1·56 fold only. Some of the kinetic studies undertaken with Enz A have been reported here. Our evidences do not indicate that the two enzyme fractions are different molecular forms of β-galactosidase.

Key words : β-galactosidase, *Aspergillus nidulans*, isolation.

1. Introduction

Significant studies on the inductive response and the biochemical properties of β-galactosidase in *Aspergillus nidulans* have been carried out by Paszewski *et al*[1], Gajewski *et al*[2] and Fantes and Roberts[3]. Paszewski *et al*[1] reported the presence of only one β-galactosidase in *A. nidulans*; Fantes and Roberts[3] later demon trated the existence of two molecular species of β-galactosidases. However, no attempt has been made to isolate and separate the enzyme from *A. nidulans*. In this paper, we present for the first time the results of such an attempt. The β-galactosidase-rich fraction has been partially purified.

2. Materials and methods

2.1. *Chemicals*

o-Nitrophenyl-β-D-galactopyranoside (ONPG) and *p*-nitrophenyl-β-D-galactopyranoside (PNPG) were obtained from BDH, Poole Ltd., England. *o*-Nitrophenol (ONP), *p*-nitrophenol (PNP) and Sepharose 4B-200 were purchased from Sigma Chemical Co., St. Louis, Mo., USA. DEAE-cellulose was obtained from Eastman Organic Chemicals,

Rochester, USA. Sephadex G-200 was obtained from Pharmacia Ltd., Uppsala, Sweden; bovine serum albumin (BSA) was obtained from Fluka Chemicals, and marker proteins for molecular weight determination (in the form of a Protein Calibration Kit) from Boehringer Mannheim, W. Germany. All other chemicals were of analytical reagent grade.

2.2. *Organism*

A wild strain of *Aspergillus nidulans* which grows with green conidia, belonging to the Glasgow stock of strain, was used.

2.3. *Preparation of lactose cultures*

Lactose media (about 3·1) were prepared according to the method of Pontecorvo *et al*[4] using lactose (1%) as the sole carbon source. The media were distributed in three 4-litre Haffkine flasks. The flasks were sterilized in an autoclave at 15 p.s.i. for 10 min and then inoculated with a heavy conidial suspension of *A. nidulans* prepared in sterile saline. The flasks were incubated for 4 days at 37° C for maximal enzyme induction.

2.4. *Assay of β-galactosidase activity*

The enzyme was assayed by the method described by Fantes and Roberts[3] with slight modifications. Except where indicated ONPG was used as the substrate for all experiments. The assay mixture contained the following: sodium phosphate buffer (pH 7·6, 0·05 M), 2·5 ml; $MgCl_2$ soln. (0·01 M), 0·3 ml; ONPG soln. (0·01 M), 0·3 ml; enzyme extract, 0·5 ml and water (to a total volume of 4·0 ml). The reaction was terminated by the addition of 1·0 ml of K_2CO_3 after incubation at 37° C for 20 min. Readings were taken in an ERMA photoelectric colorimeter at 420 nm using *o*-nitrophenol as standard. For PNPG, *p*-nitrophenol was used as the standard. Enzyme activity was expressed as nano moles of the respective nitrophenol liberated/min/mg protein. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 nano mole of the respective nitrophenol/min.

Protein content was determined by the method of Lowry *et al*[5] using BSA as standard.

2.5. *Molecular weight determination*

Molecular weights were determined by the method of Andrews[6] using Sephadex G-200. The gel was packed to a column height of 1·5 × 23·0 cm and equilibrated with 0·05 M sodium phosphate buffer (pH 7·6). The elution volume (V_e) of the enzymes were compared with those of marker proteins—cytochrome *c*, chymotrypsinogen, albumin *H*, albumin *R*, aldolase and ferritin. Void volume (V_0) was determined using Blue Dextran 2000. By plotting log Mol. Wt. against V_e/V_0, the molecular weights of the enzymes were determined.

3. Results

3.1. *Purification*

Isolation and partial purification of β-galactosidase from *A. nidulans* were carried out at 0-4° C.

Step I : Preparation of cell-free extracts

Wet mycelia (about 30 g) were washed well with 0·05 M sodium phosphate buffer (pH 7·0) containing 1·0 mM magnesium chloride in a cold room at 0–4° C. The mycelia were then mixed with glass powder and homogenized in a chilled mortar and pestle with the same buffer. The extract (about 100-150 ml) was then centrifuged at 15,000 × g for 15 min at 0° C in an MSE superspeed refrigerated centrifuge. The supernatant was collected and used for ammonium sulfate fractionation.

Step II : Ammonium sulfate fractionation

The bulk of the enzyme (88%) was found to precipitate between 30% and 60% $(NH_4)_2SO_4$ saturations. The supernatant from Step I (about 100 ml) was first brought to 30% $(NH_4)_2SO_4$ saturation—the amount added was calculated using the formula devised by Kunitz[7]. Addition of $(NH_4)_2SO_4$ was made with constant stirring for 30 min. The suspension was then centrifuged at 6,000 × g for 15 min. The precipitate was discarded and the supernatant was brought to 60% $(NH_4)_2SO_4$ saturation and was again stirred and centrifuged. The precipitate was collected and dissolved in about 10·0 ml of 0·05 M sodium phosphate buffer (pH 7·6)—Buffer A. The solution was then dialysed overnight against 2 litres of Buffer A containing 1·0 mM magnesium chloride.

This step was accompanied by a 1·5 fold increase in specific activity. Overnight dialysis in a cold room at 0–4° C against Buffer A containing 1·0 mM magnesium chloride resulted in no significant loss of activity as observed by Fantes and Roberts[3].

Step III : DEAE-cellulose chromatography

The dialysed enzyme solution was then loaded on to a DEAE-cellulose column (2·0 × 12·5 cm) washed according to Peterson and Sober[8]. The column was first developed with 200 ml of Buffer A and subjected to step-wise elution using 0·1 M, 0·2 M and 0·3 M NaCl solutions prepared in the same buffer. Eight ml fractions were collected at a flow rate of 1·0 ml/min. Two peaks of β-galactosidase activity (Fig. 1) were observed—one in the 0·2 M NaCl eluted fraction (designated as Enzyme A) and the other in the 0·3 M NaCl eluted fraction (designated as Enzyme B). Enzyme A formed the major fraction and possessed high β-galactosidase activity. The tubes containing maximum Enzyme A activities were pooled together and subjected to further purification.

Enzyme A and Enzyme B constituted 84% and 6%, respectively of the total eluted activities. The yield of Enzyme A was 47% while that of Enzyme B was 8·6%.

Step IV: *Chromatography on Sepharose 4B-200*

Enzyme A was layered on to a Sepharose 4B-200 column (1·5 × 22·0 cm) equilibrated with Buffer A. The column was eluted with this buffer at a flow rate of 20 ml/h and 3·0 ml fractions were collected. Fractions with high Enzyme A activity (Fig. 2) were pooled together and used for kinetic studies.

The yield of Enzyme A was 19·5% and exhibited a 10·7 fold increase in activity.

The complete purification data are presented in Table I.

3.2. *Molecular weight determination*

Enzyme A and Enzyme B had molecular weights of 450,000 and 320,000, respectively.

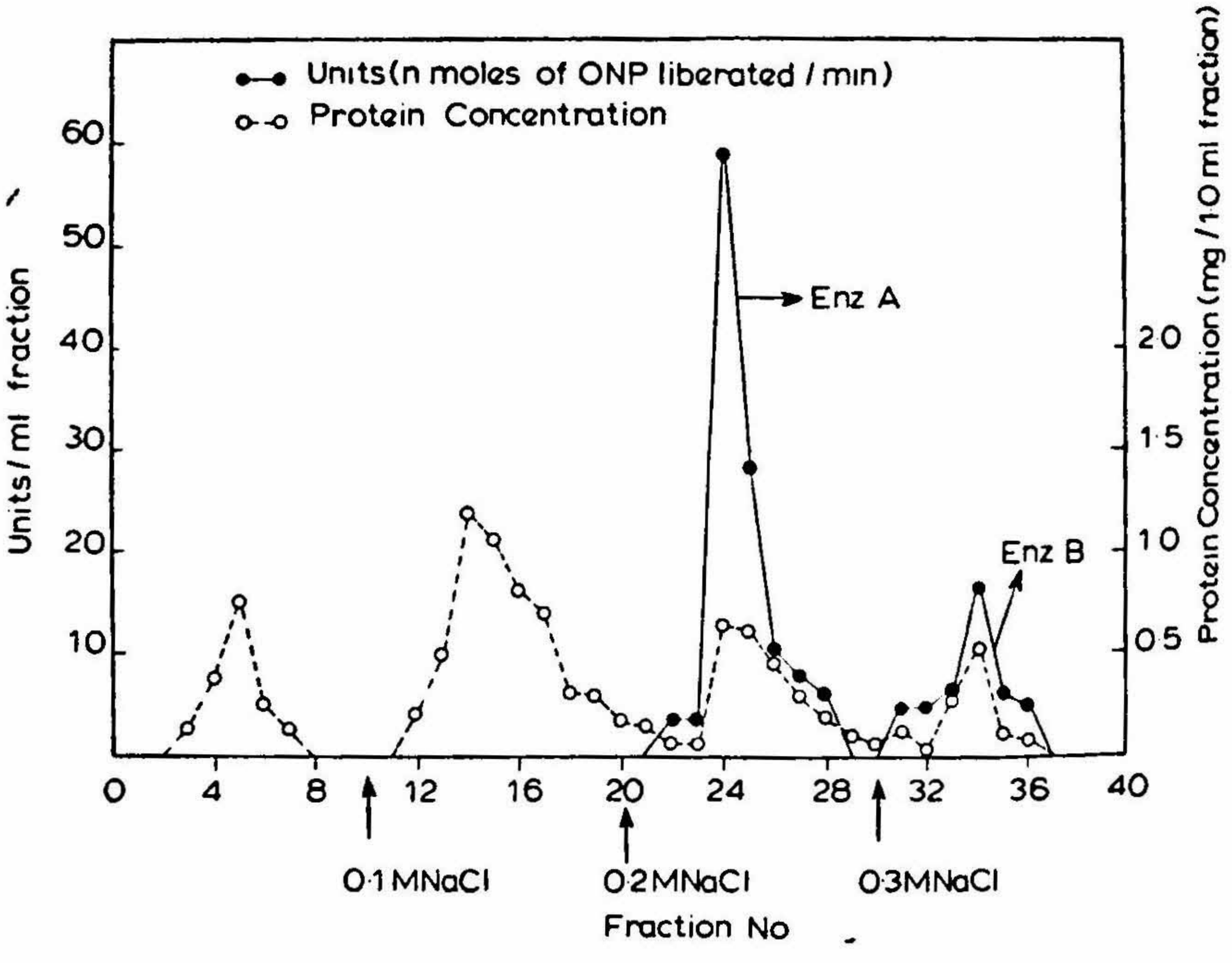

FIG. 1. DEAE-cellulose elution pattern of beta-galactosidase from *A. nidulans*.

The enzyme solution, after $(NH_4)_2SO_4$ treatment, was dialysed and loaded on to a DEAE-cellulose column (2·0 × 12·5 cm) previously equilibrated with 0·05 M sodium phosphate buffer (pH 7·6). The column was subjected to step-wise elution using 0·1 M, 0·2 M and 0·3 M NaCl solutions. Eight ml fractions at a flow rate of 1·0 ml/min were collected.

3.3. *pH and temperature dependence of the enzymes*

Both enzymes exhibited maximum activity in the pH range 7·6–7·8 and at temperatures between 37° C–40° C.

3.4. *Kinetic properties*

The apparent Km values of the enzyme (Enzyme A) for the substrates—ONPG and PNPG— were determined by using the Lineweaver–Burk plots (Figs. 3 and 4). The Km values of Enzyme A for ONPG and PNPG were $1 \cdot 82 \times 10^{-3}$ M and $3 \cdot 33 \times 10^{-3}$ M, respectively ; in the presence of 1·0 mM lactose, the Km values with the two substrates were $2 \cdot 22 \times 10^{-3}$ M and $4 \cdot 00 \times 10^{-3}$ M, respectively; with 10·0 mM lactose, they were $2 \cdot 86 \times 10^{-3}$ M and $5 \cdot 00 \times 10^{-3}$ M, respectively. It is clear that the enzyme has greater affinity for ONPG than for PNPG.

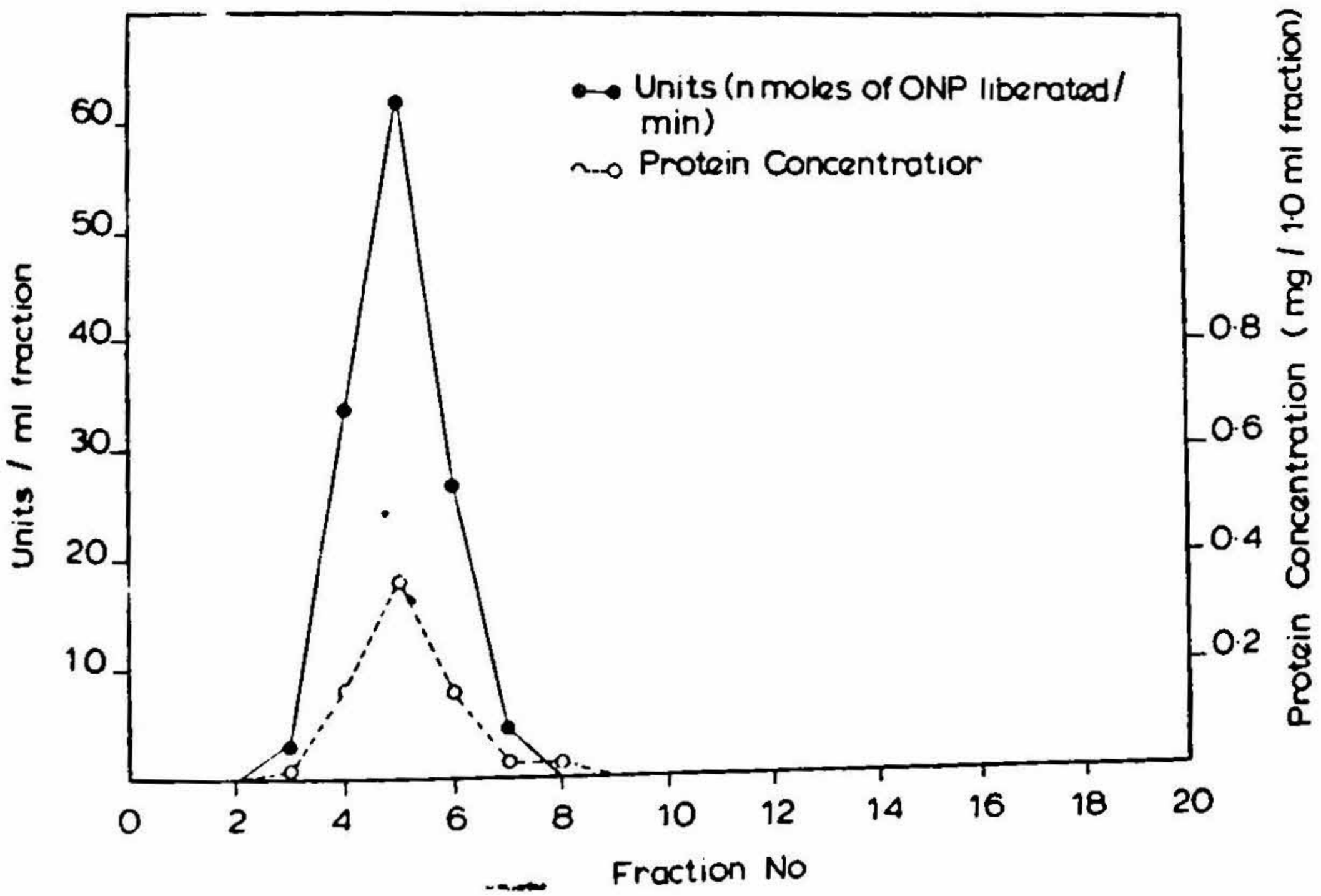

FIG. 2. Sepharose 4B–200 elution profile of Enzyme A obtained from DEAE-cellulose. Enzyme A from the DEAE-cellulose step was layered on to a Sepharose 4B-200 column (1·5 × 22·0 cm) equilibrated with 0·05 M sodium phosphate buffer (pH 7·6). The column was eluted with this buffer at a flow rate of 20 ml/hr and 3·0 ml fractions were collected.

Kinetic data on Enzyme B have not been presented because of the low fold of purification.

Table I

Summary of the purification of β-galactosidase from *A. nidulans*

Step	Purification	Vol. (ml)	Total units	Total protein (mg)	Specific activity	% Yield	Fold purification
I	Crude extract	100	2953·2	184·00	16·05	100·00	—
II	(30%–60%) $(NH_4)_2SO_4$ fractionation	10	2594·4	107·70	24·08	87·85	1·50
III	DEAE-cellulose chromatography						
	(i) 0·2 M NaCl—Enz A	32	1397·8	15·67	89·20	47·33	5·56
	(ii) 0·3 M NaCl—Enz B	24	254·3	10·16	25·03	8·61	1·56
IV	Sepharose 4B-200 chromatography of Enz A	9	576·9	3·35	172·20	19·53	10·73

Specific activity—*n* moles of ONP liberated/min/mg protein ; 1 unit is defined as the amount of enzyme that liberates 1 *n* mole of ONP/min under incubation conditions.

4. Discussion

Resolution of β-galactosidase of *A. nidulans* on DEAE-cellulose revealed two peaks of enzyme activity—designated as Enyzme A (Enz A) and Enzyme B (Enz B). Enzyme A was the major fraction possessing maximum β-galactosidase activity. The molecular weight of Enzyme A (450,000) coincided with one of the enzymes isolated by Fantes and Roberts[3] but was different from that detected by Paszewski *et al*[1] since the only enzyme detected by them had a molecular weight of 150,000. A study of the Michaelis constants indicated that Enzyme A had a two-fold greater affinity for ONPG relative to PNPG. Lactose was found to be a competitive inhibitor of the hydrolysis of ONPG and PNPG possibly because of its apparent structural similarity to both these substrates. Studies with Enzyme B were, however, precluded because of the low fold of purification. The apparent Km values of Enzyme A for ONPG and PNPG were found to be $1·82 \times 10^{-3}$ M and $3·33 \times 10^{-3}$ M, respectively. The Michaelis constants of the β-galactosidase of *A. nidulans* for either of these substrates (or of any other) have not been reported so far.

Data available from our work did not indicate that Enzymes. A and B were two distinct molecular forms of β-galactosidase. On the other hand, Enzyme B could very well be an artifact of the major enzyme, Enzyme A, for the following reasons:

(i) Step-wise elution, as the one adopted by us, is known to result in artifacts of enzyme simulating enzyme activity in discrete fractions[9].

(ii) A study of the Km values of Enzymes A and B, after DEAE-cellulose chromatography, revealed no significant variations (data not presented).

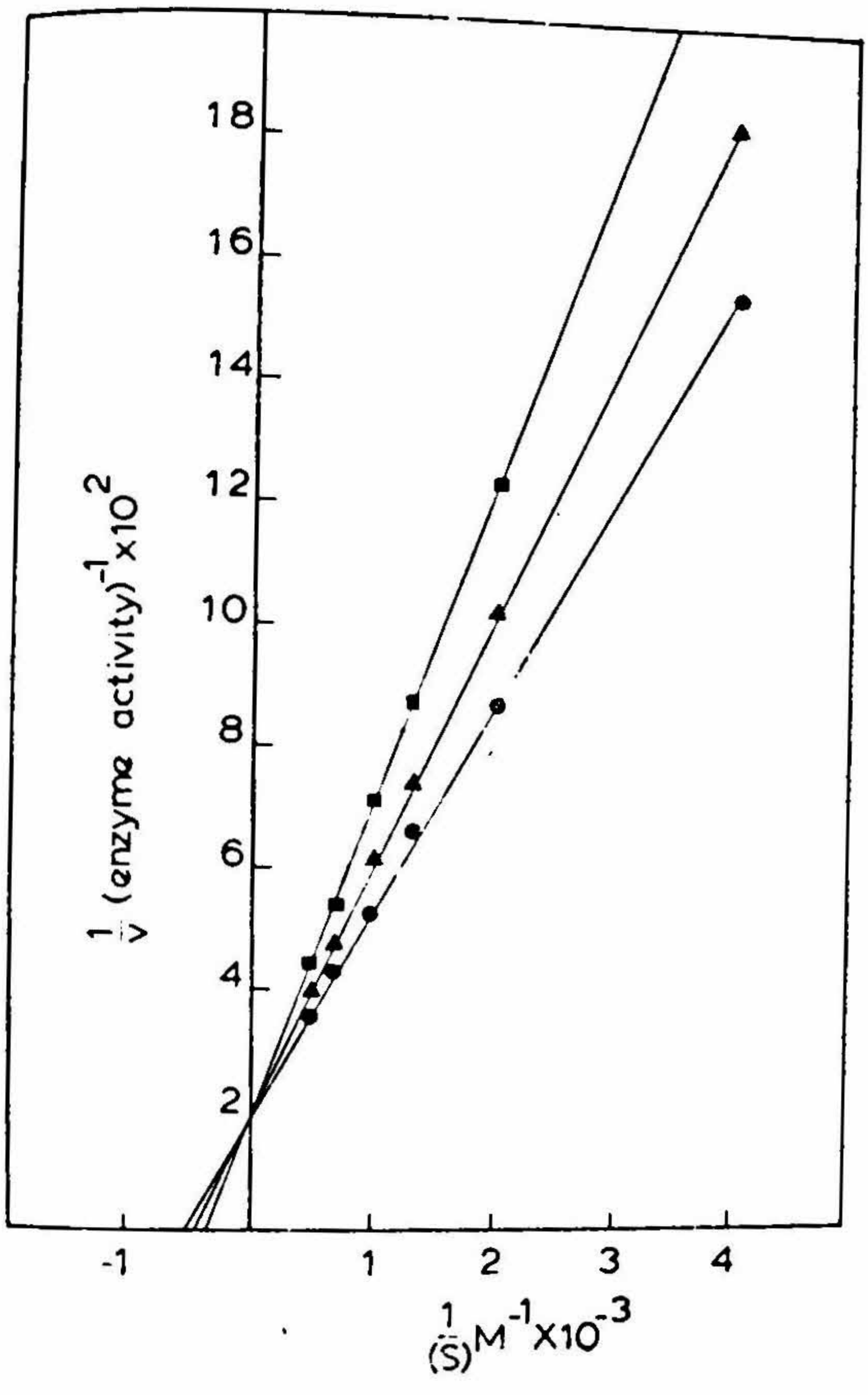

FIG. 3. Lineweaver-Burk plot of Enzyme A using ONPG as substrate (●—● ONPG as substrate; ▲—▲ ONPG + 1·0 mM lactose ; ■—■ ONPG + 10·0 mM lactose).

(iii) Both enzymes exhibited similar pH and temperature optima.

(iv) The molecular weight difference between Enzymes A and B (450,000 and 320,000, respectively) appeared insignificant when their elution volumes on Sephadex G-200 (17 ml and 19 ml, respectively) were considered.

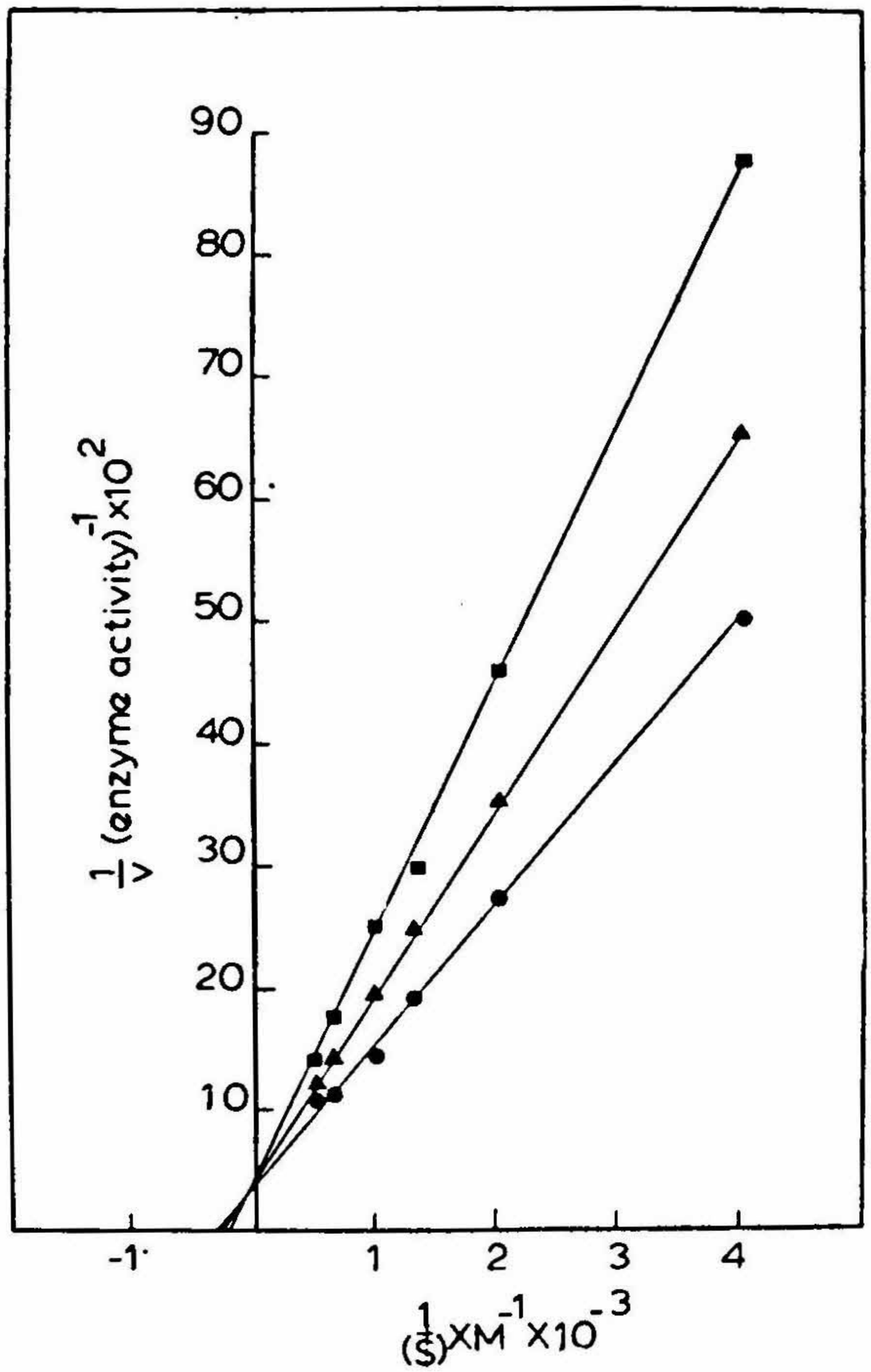

FIG. 4. Lineweaver–Burk plot of Enzyme A using PNPG as substrate (●—● PNPG as substrate; ▲—▲ PNPG + 1·0 mM lactose; ■—■ PNPG + 10·0 mM lactose).

This appears to be the first and only report on the isolation and separation of β-galactosidase from *A. nidulans*. Attempts in increasing further the purity and yield of the enzyme by other purification techniques are in progress.

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References

1. PASZEWSKI, A., CHOJNACKI, T., LITWINSKA, J. AND GAJEWSKI, W. — Regulation of lactose utilization in *Aspergillus nidulans*, *Acta Biochim. Pol.*, 1970, 17, 385–391.

2. GAJEWSKI, W., LITWINSKA, J., PASZEWSKI, A. AND CHOJNACKI, T. — Isolation and characterization of lactose non-utilizing mutants of *Aspergillus nidulans*, *Mol. Gen. Genet.*, 1972, 116, 99–106.

3. FANTES, P. A. AND ROBERTS, C. F. — β-galactosidase activity and lactose utilization in *Aspergillus nidulans*, *J. Gen. Microbiol.*, 1973, 77, 471–486.

4. PONTECORVO, G., ROPER, J. A., HEMMONS, L. M., MACUCONALD, K. D. AND BUFTON, A. W. J. — The genetics of *Aspergillus nidulans*, *Adv. Genet.*, 1953, 5, 141.

5. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. AND RANDALL, R. J. — Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 1951, 193, 265–275.

6. ANDREWS, P. — The gel-filtration behaviour of proteins related to their molecular weights over a wide range, *Biochem. J.*, 1965, 96, 595–606.

7. KUNITZ, M. — Crystalline inorganic pyrophosphatase isolated from baker's yeast, *J. Gen. Physiol.*, 1952, 35, 423–450.

8. PETERSON, E. A. AND SOBER, H. A. — Column chromatography of proteins : substituted celluloses, *Methods in enzymology*, Vol. 5, Eds.: Colowick, S. P. and Kaplan, N. O., Academic Press, Inc., New York, 1962, pp. 3–27.

9. ALM, R. S., WILLIAMS, R. J. P. AND TISELIUS, A. — Gradient elution analysis. I. A general treatment, *Acta Chem. Scand.*, 1952, 6, 826–836.