PURIFICATION OF EXOINULINASE *BIFIDOBACTERIUM BREVE*

(PEMURNIAN EKSOINULINASE DARI *BIFIDOBACTERIUM BREVE)*

Pudjono*) and G. Barwald **)
*) Faculty of Pharmacy, Gadjah Mada University, Sekip Utara, Yogyakarta
**)Department of Food Science and Biotechnology Technical University of Berlin, Seestr. 13, D-13353 Berlin, Germany

ABSTRACT

Extracellular exoinulinase was produced from *Bifidobacterium breve* in media with inulin as the only C-source. The enzyme was purified on DEAE-Sephadex A-50, preparative IEF and gel filtration yielded a purification of 68-fold. The pI range was from 4.2 to 4.9, the molecular weight was identified using polyacrylamide gel electrophoresis (SDS-PAGE) and the result was 64; 59 and 32 kD. It did not hydrolyse inulin into smaller fructan units or sucrose, therefore it was characterized as an exo-type enzyme.

Key words: Bifidobacterium, exoinulinase, inulin

ABSTRAK

Eksoinulinase ekstraseluler dihasilkan dari *Bifidobacterium breve* dalam media inulin sebagai satu-satunya sumber karbonnya. Enzim dimurnikan menggunakan DEAE Sephadex A-50, IEF Preparative, filtrasi gel, dan diperoleh kemurnian 68 kali. Harga pI yang diperoleh berkisar dari 4,2 sampai 4,9 dengan massa molekul berturut-turut: 64, 59 dan 32 kD yang diuji dengan poliacrilamide gel elektroforesis (SDS-PAGE). Enzim tidak dapat menghidrolisis inulin kedalam unit-unit fruktan atau sukrosa, sehingga diperoleh suatu kesimpulan bahwa ensim tersebut dikarakterisasikan sebagai tipe eksoensim.

Kata Kunci: Bifidobacterium, eksoinulinase, inulin

INTRODUCTION

Bifidobacteria, main constituents of normal intestinal flora of man, are considered to prevent the man from some bacterial disease. Breast-fed infants have bifidus-flora in their intestines where bifidobacteria are predominant over other bacteria, while bottle-fed infants have a flora where the number of bifidobacteria decreases or that of other bacteria increases (Yazawa et al., 1978). Exo-inulinase is a enzyme, which hydrolyzes not only sucrose but also inulin vary widely. In a former research about fructan hydrolytic enzymes we have reported that the production of inulinase and invertase from *Bifidobacterium adolescentis* (Pudjono *et al.*, 1993). *B. breve* was found in the faeces of human adults and in the human intestine. Fructan hydrolytic enzymes were isolated from plants (Gupta *et al.*, 1993), from fungi (Xiao *et al.*, 1988; Barthomeuf *et al.*,1991; Kim and Rhee, 1989; Matsuyama and Tanaka, 1989), from yeast (Rouwenhorst *et al.*, 1990; Nahm and Byun, 1977; Bajpai and Margaritis,1982) and from bacteria (Uchiyama,1975 and Seki *et al.*, 1989).

METHODS

In this study *B. breve* (ATCC 15700) was used. The medium for the enzyme production was a modification of the Bifidobacterium medium, that 1% glucose was substituted with 2% inulin. The precultivation was made anaerobic using a CO_2 atmosphere at 37°C for 48 h. This culture was inoculated into 1-5 L of the same medium until a concentration of 10^6 cells/ml were obtained. The fermentor was adjusted at 37°C, stirred with a rate of 100 rpm, and anaerobic conditions were made using a gas flow 0.1 ml of N_2/min. The pH was controlled and adjusted to 6.8 with 5 N KOH. When the acids production were finished the fermented media were collected. The microorganisms were removed by centrifugation at 4,500

x g for 20 minutes. The supernatant was concentrated through a minisette ultrafiltration membrane (30 kD cut off, Filtron). The concentrated preparation from the supernatant was used as a starting material for the purification of the extracellular enzymes.

Enzyme assay: Inulinase activity was calculated by determining the released of fructose. The reaction mixture contained 0.9 ml of 1.0% inulin in 0.1 M phosphate buffer pH 6.5 and 0.1 ml of enzyme solution. The mixture was incubated at 45°C in a water bath for 30 minutes. Then it was heated at 90°C in a water bath for 3 minutes, and bidestill. water was added to 10,0 ml. The free fructose was determined by enzymatic test kit (Boehringer, Mannheim). One unit U of the inulinase activity was defined as (one)1 mole of fructose produced per minute in above conditions. Invertase activity was calculated by determining the released of free sugars as described above with 1.0% sucrose in 0.1 M phosphate buffer pH 6.5 as substrate. One unit U of the invertase activity was defined as 1 µ mole of sucrose hydrolysed per min. in above conditions.

Determination of protein: Protein was determined by the biuret method using bovine serum albumin as a standard. The absorbance at 280 nm was used for monitoring protein in column eluates.

RESULTS AND DISCUSSION

Purification of extracellular enzymes: The concentrated preparation from the supernatant was dialyzed against bidest.water. The dialyzed solution was concentrated by lyophilisation, after resolving was absorbed on a DEAE- Sephadex A-50 column equilibrated with 10^{-2} M phosphate buffer, pH 5.6.

Then the enzyme was eluted with sodium chloride gradient concentrations (0.05-0.5 M) in the same buffer. The protein concentration was measured at 280 nm. The active fractions were collected, lyophilized and used for preparative isoelectro focussing with Sephadex G-200. The results are shown in Figure 1 and Table I

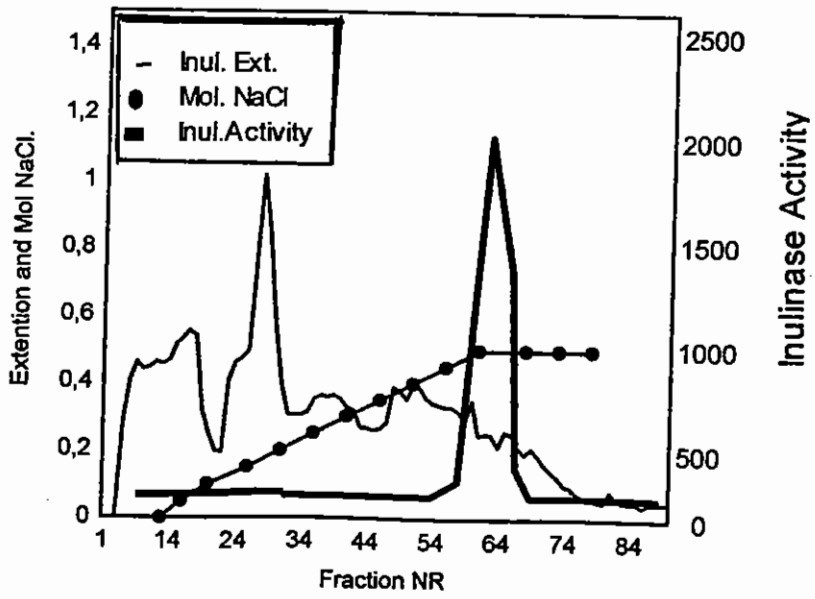

Fig.1: Chromatography of the extracellular enzyme on DEAE-Sephadex A-50

The inulinase activity of fraction V was 68 fold of that crude protein. Gelfiltration with sephadex G-100: Further the active fractions (III, IV and V) were lyophilized and fractionated on sephadex G-100

column (120x1.2 cm) with 50 mM phosphat buffer pH 6.0. Fraction volumes were 5.0 ml with an elution rate of 10 ml per hour.

Table I: Isoelectric focusing of active fractions after separated by anion exchange chromatography with DEAE-Sephadex A-50

Fract.Nr.	Protein (mg)	pI	Specific activity of			
			Inulinase	Conc.%)	Invertase	Conc (%)
I	40.5	5.95	0.2	0.02	3.6	0.45
II	36	5.34	0.7	0.07	9.3	1.15
III	32.2	4.96	92.4	9.41	211.3	26.23
IV	36	4.56	408.7	41.60	565.5	70.61
V	27	4.25	478.8	48.73	12.3	1.53
VI	24	3.94	1.7	0.17	3.5	0.43

The results are shown in Figure 2. only the fractions III and V show a separation effect of both enzymes: exoinulinase and invertase. Both fractions were investigated for the determination of the molecular weight using polyacrylamide gel electrophoresis (SDS-PAGE) in a phase system (Pharmacia). The results are shown in Figure 3.

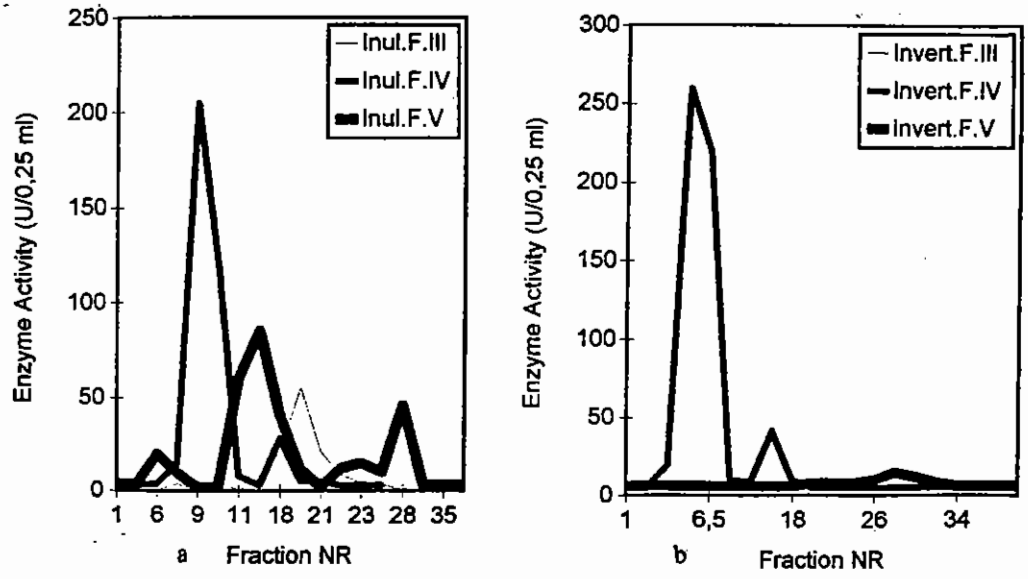

Fig.2: Chromatographs of fractions III, IV and V after the gel filtration on sephadex G-100 a. Inulinase and b. Invertase.

Effects of activators and inhibitors: The effects of metal ions on the purified enzyme were investigated and the results are shown in Table II.

As shown in Table II only Hg^{2+} inhibited both the activity of inulinase and invertase, whereas Zn^{2+} and Fe^{3+} inhibited little on the activity of invertase, but on the other hand Mn^{2+} stimulated both on the inulinase activity and the invertase activity.

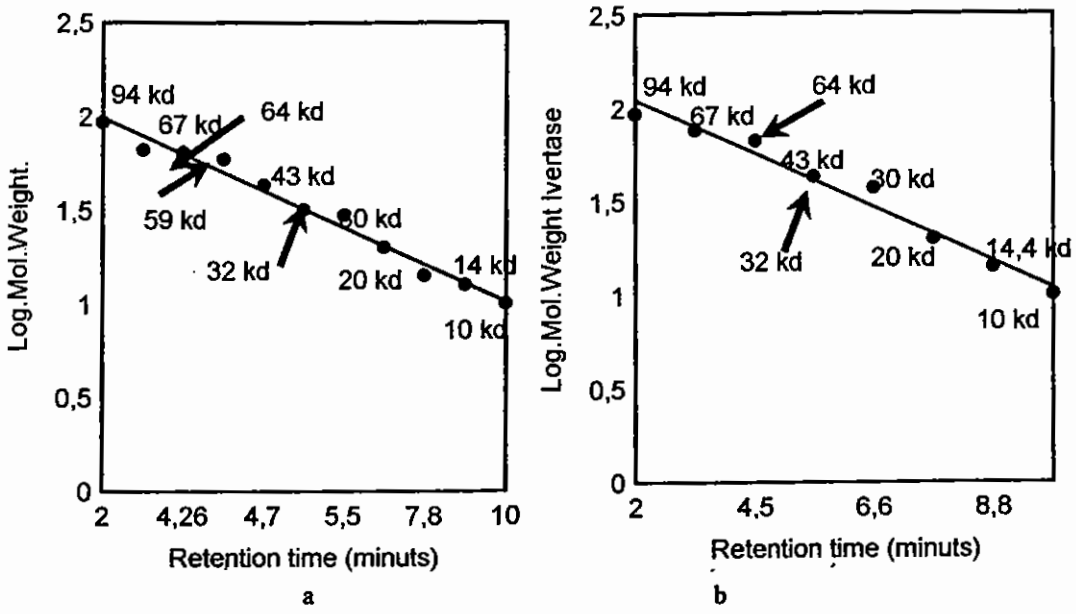

Fig.3: Estimation of the MW of the purified protein and fractions which show enzymatic activitiesby SDS-PAGE. a Inulinase b. Invertase

Table II: Effects of 1 mM activating respons inhibiting substances of inulinase and invertase activities.

Compound	Relative activity (%)	
	Inulinase	Invertase
Control- (H_2O)	100	100
$CaCl_2$	105.1	97.4
$MgCl_2$	104.6	93.4
$MnSO_4$	154.3	133.2
$ZnSO_4$	99.0	76.1
$HgCl_2$	6.4	6.0
$FeCl_3$	92.1	84.6

Effects of pH: The activity of purified inulinase was measured at various pH from 3.0 to 8.0 (0.1 M citrat buffers between pH 3.0-5.0 and 0.1 M phosphate buffers between pH 5.2 to 8.0). As shown on Fig. 4a, the optimal pH for inulinase was pH 6.5

Effects of temperature on the activity of inulinase: The effects of temperature on the inulinase activity was examined at 12 different temperature in the range from 20°C to 75°C. As shown in Fig. 4b, the inulinase activity has an optimum temperature at 45° C.

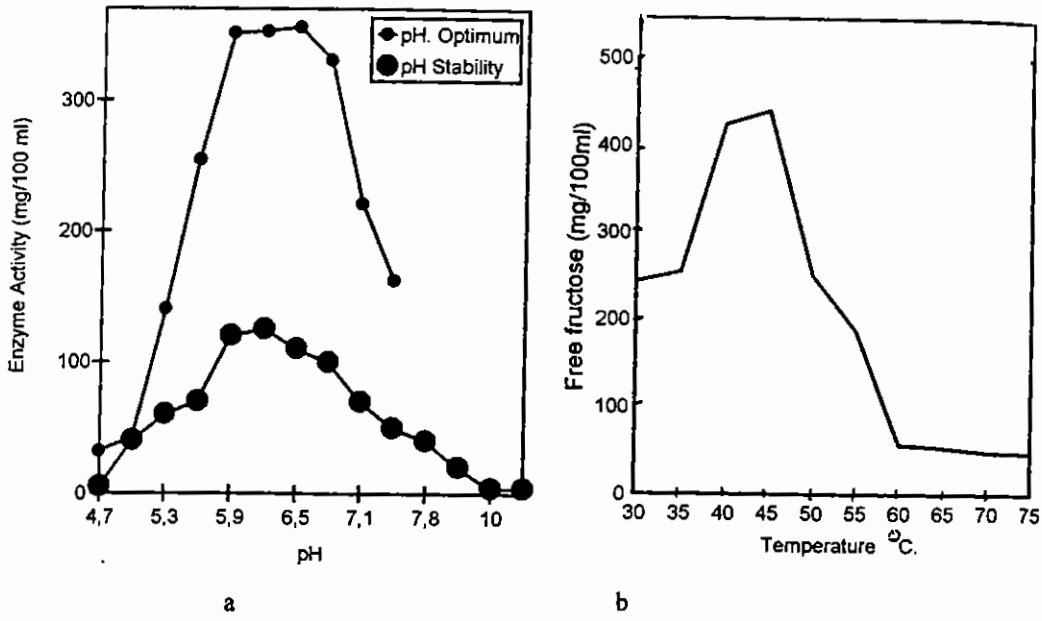

Fig.4: Curve of a. Effect of pH on the inulinase activity, calculated with the released fructose, and pH-stability b. Effect on temperature (^{0}C)

Inulinase purified from the medium was exoinulinase, because in thin layer chromatography only one spot from fructose was detected. The molecular weights of this inulinase were found at 64 kD, 59 kD and 32 kD with Km =0.2 mM (MW inulin=5040 kd) whereas MW from invertase was found 76 kD and 38 kD (Fig. 3) with Km = 0.14 mM. As shown in Table I, the inulinase activity was highest in fraction V, whereas in fraction IV there a mixture was mixing between invertase and inulinase. The invertase activity was highest in fraction III. From gel filtration (Fig. 2) was shown that the fraction V contained 3 peaks with inulinase activity, fraction IV only 2 peaks from inulinase and invertase could be detected, and in fraction III there were one large peak of invertase and a little one of inulinase. Both enzymes were inhibited strongly by Hg^{2+} and activated strongly by Mn^{2+}

On the other hand invertase was inhibited little by Zn^{2+}. Inulinase had an optimum temperature at 45°C (Fig. 4b), this activity lost at 60°C and the inulinase activity was decreased until 89% at 75°C. Whereas the inulinase activity had optimum at pH 6.5 (Fig. 4a) and this enzyme was stable in a pH range between 4.5 and 11.0 (Fig. 4a).

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