Indian Journal of Experimental Biology
Vol. 18, September 1980, pp. 1020-1023

Immobilization & Characterization of *Kluveromyces fragilis* β–Galactosidase

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Manuscript received 4 *December* 1979; *revised manuscript received* 1 *May* 1980

Cells of *K. fragilis* and soluble β-galactosidase were immobilized on fibrous collagen and their properties were compared with the soluble enzyme. There was a shift of *p*H optimum from 7.0 to 6.6 when the enzyme and the cells were immobilized on collagen. Immobilization did not change the optimum temp. (35°C) requirement of the enzyme but the thermal stability of the enzyme was increased. Mg^{2+}, Mn^{2+} and Co^{2+} increased the enzyme activity whereas Hg^{2+} and Cu^{2+} completely inhibited it. Kinetic data for the free and bound enzyme were determined using lactose and ONPG as substrate. Among the lactose containing substrates tried, the extent of lactose hydrolysis was maximum in lactose buffer solution. The milk in which 34% of lactose was hydrolysed was used for the preparation of yoghurt. The characteristics and acceptability of yoghurt were not affected by the treatment of milk.

A variety of β-galactosidase have been extensively studied for possible use in the preparation of lactose reduced milk and milk products, intended for use by lactose intolerant persons. But the application of the enzyme on industrial scale has been limited due to high cost of soluble enzyme. Use of enzyme in an immobilized form could give significant economic advantages. The process can be made more profitable by use of immobilized microbial cells as an enzyme source. This will eliminate the extraction and purification steps required for the utilization of the enzyme.

Immobilization of β-galactosidase of *Escherichia coli*, *Aspergillus niger* and *Saccharomyces lactis* have been reported[1-3]. Although *Kluveromyces fragilis* was reported to be the best β-galactosidase producer for use in food industry[4-6] very little work has been done on the immobilization of β-galactosidase on collagen from the same organism. We, therefore, report the immobilization of soluble β- galactosidase and *K. fragilis* cells on fibrous collagen. The kinetics and properties of soluble and bound enzyme and immobilized whole cells have been studied.

Materials and Methods

K. fragilis Y - 1109 was obtained from Northern Regional Research Laboratory, USA. The comminuted hide collagen is a research product of Eastern Regional Research Centre, Philadelphia. Yeast was maintained on Wendorff and Amundson[7] medium, with the following composition (g/l): lactose, 10; yeast extract, 5; K_2HPO_4, 3; $(NH_4)_2SO_4$, 1; and *p*H 5.5. The culture was incubated at 30°C for 72 hr as a shake culture. The yeast cells were harvested by filtration and washed in phosphate buffer (0.2*M*, *p*H7). The cell suspension was employed for immobilization as early as possible.

Isolation of the enzyme — The yeast cells were disrupted in a mortar with alumina and 10 ml of toluene: acetone (9:1) mixture. Phosphate buffer (10ml) was then added and centrifuged at 10,000 for 15 min. The clear supernatant was used as the crude enzyme. The crude extract was purified by ammonium sulphate fractionation and column chromatography on Sephadex G-200. The purified enzyme having a sp. activity of 112 units was used for immobilization.

Immobilizations — The yeast cells and free enzyme were immobilized on collagen fibres[8]. The lyophilized fibrous collagen was allowed to swell in phosphate — magnesium buffer containing the free enzyme or microorganism for 3 hr at 4°C. Glutaraldehyde was then added to give final glutaraldehyde conc. of 0.5% and the cross linking allowed to continue for 15 min. The collagen enzyme or cell complex was separated from supernatant by filtration and washed repeatedly with phosphate buffer. For determination of enzyme activity of collagen enzyme or cell complex, 300 mg of cells/g of collagen or 1680 enzyme units/ g of collagen were used in place of the soluble enzyme and proceed in same way as for the soluble enzyme.

Enzyme assay — The enzyme was assayed using *o*-nitrophenyl β-D-galactopyranoside as substrate. The amount of *o*-nitrophenyl released was determined spectrophotometrically at 420 by the procedure described by Lederberg[9].

Effect of flow rate and repeat runs on lactose hydrolysis — Five g of collagen — whole cell complex was used in a column (2 x 30 cm) fitted with sintered glass which served as support for immobilized whole cells. Lactose solution (5%) was passed through the column of immobilized cells and different flow rates from 20-100 ml/hr were tried. Two ml aliquots were analysed for glucose content by the glucose oxidase — peroxidase procedure[10].

To study the effect of repeat runs, 50 ml fractions of 5% lactose solutions were passed through the column of immobilized cells. The columns were given washing in between two runs using 25

0.2 *M* phosphate buffer. Appropriate aliquots were analysed for enzyme activity. The time for each repeat run was 2.5 hr.

Effect of various lactose containing substrates — Four per cent lactose solution, whey, skim milk and whole milk were incubated with a known amount of immobilized whole cells at 37°C for 15 min and the reaction mixture was assayed for enzyme activity. The lactose content was estimated as per the gravimetric method[11].

Preparation of yoghurt — Lactose (34%) in milk was hydrolysed by treatment with the immobilized cells. The treated milk was then used for preparation of yoghurt[12].

Results

Relationship between amount of enzyme offered and enzyme bound — The purified enzyme solution was added to give 2240, 3360, 4480 and 5600 units per g of collagen for binding. The amounts of enzyme bound increased with the increase in the amount of the enzyme added, the maximum being 1680 units per g collagen when 5600 units were offered (Table 1). On the other hand, coupling efficiency which is an expression of the efficiency of binding of enzyme to collagen, decreased with the increase in enzyme units added.

Collagen — enzyme complex containing 1680 enzyme units per g of collagen, was used in subsequent studies.

Effect of pH and temp. on enzyme activity — The maximum activity for the free enzyme occurred at pH 7 whereas the optima for collagen bound β-galactosidase and immobilized yeast cells were at pH 6.5. Immobilization did not change the optimum temp. (35°C) requirement of the enzyme.

Thermal stability — The free and bound β-galactosidase and immobilized cells were incubated in phosphate buffer at different temp. (30°-35°C) for 30 min. The residual activity was measured. The immobilized β-galactosidase and cells were more stable than the corresponding free enzyme at higher temp. (40°-55°C). They retained 100 per cent of initial activity at 40°C whereas the free enzyme showed only 83% of the initial activity (Fig. 1).

Effect of metal ions — The free and bound β-galactosidase and immobilized cells were incubated at 37°C for 15 min in ONPG solution containing various metal ions (Table 2). Heavy metal ions Hg^{2+} and Cu^{2+} completely inhibited the enzyme activity. Ca^{2+}, Fe^{2+} and Ba^{2+} also caused slight decrease in enzyme activity. The divalent cations Mn^{2+}, Mg^{2+} and Co^{2+} enhanced the enzyme activity. However, the immobilized enzyme and immobilized cells were found to be more sensitive to the inhibitory action by various metal ions.

Enzyme kinetics — All the 3 enzyme preparations obeyed Michaelis — Menton Kinetics towards ONPG and lactose. Line weaver — Burk plots were drawn and K_m values calculated. The apparent K_m values for the immobilized enzyme and the immobilized cells were slightly higher than that of the free enzyme (Table 3).

Effect of flow rate on lactose hydrolysis — Lactose solutions (5%) were passed through a column of collagen *K. fragilis* cell complex. When flow rate was 20 ml/hr, 68% of lactose was hydrolysed. It decreased to 54,52 and 35% when the flow rate was increased to 50,80 and 100 ml/hr, respectively.

Effect of repeat runs on lactose hydrolysis — The steady limit activity was attained after the first repeat run (Fig. 2). The activity at steady state was equivalent to 44 % of the initial activity.

Effect of lactose containing substrates — Among the lactose containing substrates tried, the extent of lactose hydrolysis was maximum (54%) in the

TABLE 1 — COUPLING EFFICIENCY AS FUNCTION OF ENZYME OFFERED

Activity offered (Total units)	Units bound/g of collagen	Coupling efficiency
2240	941	42
3360	1278	38
4480	1568	35
5600	1680	30

Substrate for assay, ONPG; Temp., 28°C; Buffer, 0.2 *M* phosphate

TABLE 2 — EFFECT OF VARIOUS METAL IONS ON β-GALACTOSIDASE ACTIVITY

Metal	Conc. (*M*)	Residual activity: Free enzyme	Immobilized enzyme	Immobilized cell
Control	0	1.00	1.00	1.00
Hg^{2+}	10^{-4}	0.04	0.00	0.00
Mg^{2+}	10^{-4}	1.13	1.11	1.05
Ca^{2+}	10^{-4}	0.93	0.80	0.78
Cu^{2+}	10^{-5}	0.06	0.00	0.00
Ba^{2+}	10^{-5}	1.06	0.82	0.94
Mn^{2+}	10^{-5}	1.66	1.53	1.55
Fe^{2+}	10^{-5}	0.83	0.82	0.72
Co^{2+}	10^{-5}	1.20	1.05	1.11

Substrate for assay, ONPG; Temp. 37°C

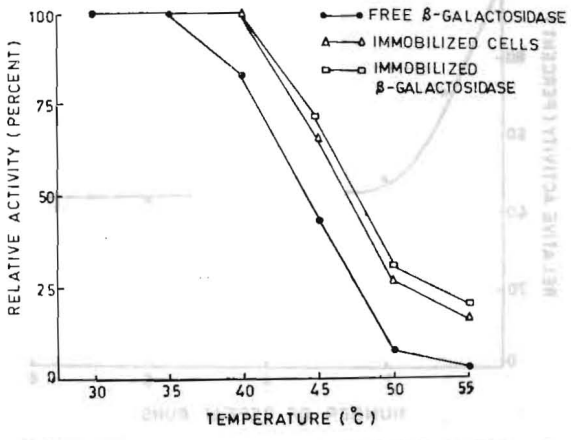

Fig. 1 — Thermal stability of β-galactosidase

lactose buffer solution and least (32%) in the whole milk (Table 4).

Preparation of yoghurt — Lactose (34%) in milk was hydrolysed by passing milk through the column of immobilized cells and the treated milk was used for the preparation of yoghurt. Characteristics of the yoghurt like titratable acidity and volatile fatty acids were not affected by lactose hydrolysis. On the other hand, the yoghurt showed slightly improvement in these characteristics (Table 5). It is spectacular to note that the new product possessed highly acceptable flavour and taste.

Discussions

Comminuted hide collagen was reported to be an excellent support for immobilized enzyme[8]. When fibrous collagen is kept in buffered enzyme or cell solution, non-covalent bonding of enzyme or cell to collagen fibres occurs. But these bonds are weak and can be easily washed away. The cross linking agents, glutaraldehyde, ties down the enzymes or cells which are already bound. In the present study, by using glutaraldehyde cross linking method, a collagen enzyme complex was obtained which contained appreciable amount of enzyme activity (1680 units/g complex). The amount of enzyme bound was not proportional to the amount of enzyme added.

TABLE 3 — MICHAELIS CONSTANTS FOR β-GALACTOSIDASE OF *K. fragilis*

	Lactose $K_m(10^{-3}\ M)$	ONPG $K_m(10^{-3}\ M)$
Free β-galactosidase	13.33	1.37
Immobilized-β-galactosidase	16.67	1.79
Immobilized cells	15.38	1.63

TABLE 4 — INFLUENCE OF VARIOUS LACTOSE CONTAINING SUBSTRATES ON THE RATE OF HYDROLYSIS OF LACTOSE

Substrate[a]	Initial lactose content (%)	Lactose hydrolyzed[b] (%)
Lactose solution	4.0	54
Whey	3.6	37
Skim milk	4.0	34
Whole milk	4.2	32

[a]*p*H of all substrates were adjusted to 6.5
[b]Per cent of lactose hydrolysed was calculated from the amount of glucose produced

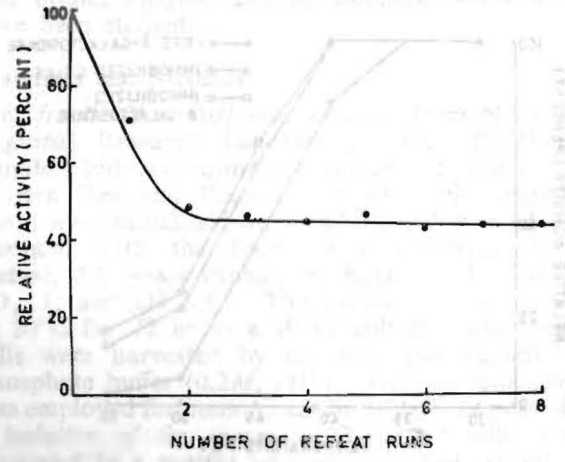

Fig. 2 — Effect of number of repeat runs on enzyme activity of collagen cell complex

TABLE 5 — CHARACTERISTICS OF YOGHURT PREPARED FROM MILK TREATED WITH IMMOBILIZED *K. fragilis* CELLS

Characteristic	Yoghurt prepared from untreated milk	Yoghurt prepared from treated milk (lactose hydrolysed 34%)
Titratable acidity[a]	0.789	0.766
Volatile fatty acids[a]	0.042	0.044
Taste and flavour[b]	+++	+++
Acceptability[b]	+++	+++

[a]Expressed as per cent w/v
[b]Result of organoleptic tests by a panel of 5 judges.

The increased stability of the immobilized enzyme and cells at acidic *p*H values might be attributed to micro-environmental effects such as the proximity of the charged groups of the matrix. This shows that the matrix is positively charged. Husted *et al*[13] attributed the upward shift in *p*H optimum to negatively charged matrix. The rate of the reaction was higher for the immobilized enzyme and cells than the free enzyme when the enzymic reaction was carried out at high temp. (45°-55°C). Moreover, the thermal stability of the immobilized enzyme was also found to be increased. This could be due to restrictions on conformational flexibility of the enzyme by cross linking to a stable support. Immobilization may make the properties of the enzyme more similar to those *in vivo* state where the enzyme is in a highly confined and restricted environment and is greatly modified by density and proximity of other macromolecules. Ohmiya *et al.*[14] also reported that thermal stability of the immobilized cells was slightly increased by encapsulation in polyacrylamide gels.

Divalent cations (Mg^{2+}, Mn^{2+} and Co^{2+}) activated whereas the heavy metal ions inhibited the enzyme activity (Table 2). The inhibitory action was more pronounced in case of the immobilized enzyme and cells and it needs further exploration.

The high apparent K_m values for the immobilized β-galactosidase can be attributed to the diffusion limitations of the substrate between the fibres. The substrate concentration in the micro-cavities was required for diffusion of more substrate into these cavities. The active sites of the enzyme were not affected by immobilization as is evident from comparable K_m values. An increased apparent K_m of about 1.9 times was reported by Sharp *et al.*[15], who immobilized β-galactosidase on cellulose sheets. An immense correlation existed between the flow rate and the amount of lactose hydrolysed. The degree of lactose hydrolysis decreased considerably during the first and second

repeat runs. The decrease could be due to leaching of loosely bound cells from the fibres. This has partially corroborated the findings of Giacin *et al.*[16] who got steady limit activity after 5 runs on collagen - lactose complex. The type of lactose containing substrates greatly influenced the extent of lactose hydrolysis. It appeared that the non-lactose solids in milk and whey had an inhibitory effect on the enzyme activity. The present results demonstrate the feasibility of employing a collagens — *K. fragilis* cell complex to reduce lactose content of milk for the use of lactose intolerant persons.

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