Article

J. Mex. Chem. Soc. **2011**, *55(4),* 246-250

ISSN 1870-249X

Linamarase Enzyme from *Lactobacillus delbrueckii* NRRL B-763: Purification and Some Properties of a β-Glucosidase

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Received June 27, 2011; accepted September 15, 2011

Abstract. Some biochemical properties and purification of linamarase enzyme from *Lactobacillus delbrueckii* NRRL B-763 were studied. The crude enzyme was used to detoxify cassava flour cyanide and samples of 150 μm particle size treated with the crude enzyme showed a reduction from 2.1 mg HCN/10g sample to 0.11 mg HCN/10 g sample after 20 h (95% reduction). Untreated control samples of 0.5 mm particle size showed a reduction from 2.1mg HCN/10 g sample to 1.98 mg HCN/10 g sample after 40 h (5.7% reduction). The enzyme was purified 33 fold with a 40% yield through a series of four steps namely, ammonium sulphate precipitation, acetone precipitation, ion exchange chromatography and gel filteration chromatogrrarphy using Sephadex G-200. The purified enzyme showed maximum activity at pH 4.5. The enzyme showed 100% stability at the pH range of 5.0 and 6.0. Maximum activity of the enzyme was observed at a temperature of 50 ^{o}C and maximum stability at a temperature range of 40 and 50 ^{o}C. The approximate enzyme molecular weight was estimated to be 56 kDa by Sephadex G-200 gel filteration chromatography. The linamarase enzyme could be adapted for improved degradation of cassava cyanide and other biotechnological applications.
Key words: Linamarase enzyme, cassava; cyanide detoxification; *Lactobacillus delbrueckii.*

Resumen. Se estudiaron algunas propiedades bioquímicas y la purificación de la enzima linamarasa obtenida de *Lactobacillus delbrueckii* NRRL B-763. La enzima cruda fue usada para eliminar los compuestos cianogénicos de harina de yuca. Muestras de 150 μm en el tamaño de la partícula tratadas con la enzima cruda mostraron una reducción del 95% en el contenido de estos compuestos tóxicos después de 20 horas de tratamiento. Las muestras sin tratar, testigo, de 0.5 mm de tamaño de partícula de sólo el 5.7% después de 40 horas. La enzima fue purificada 33 veces con un rendimiento del 40% a través de una serie de 4 pasos, precipitación con sulfato de amonio y con acetona, sucesivamente, cromatografía en columna de intercambio iónico y cromatografía de filtración en gel con Sephadex G-200. El pH óptimo de la enzima purificada fue de 4.5. La enzima es estable 100% en el rango de pH de 5.0 a 6.0. La actividad máxima de la enzima se observó a una temperature de 50 °C. La masa molecular aproximada de la enzima es de 56 kDa calculada a partir de su tiempo de elusión de una columna de permeasión en gel con Sephadex G-200. La enzima linamarasa podría ser adaptada para mejorar la degradación de los compuestos cianogénicos de la yuca, así como para otras aplicaciones biotecnológicas.
Palabras clave: detoxificación cianogénica, enzima, *Lactobacillus delbrueckii*, linamarasa, yuca.

Introduction

Cassava (*Manihot esculenta* Crantz) is an important source of calories in tropical countries. Because of the presence of cyanoglycosides, cassava is potentially toxic to human populations that subsist on cassava-based diets. Almost all of the tissues of cassava contain large amounts of cyanogenic glycosides, linamarin and lotoaustralin [1]. Linamarin accounts for 95% of the total cyanoglycosides and most of the research on cassava cyanoglycosides has been focused on the biochemistry and metabolism of such compounds [2- 4].

Cyanide is highly toxic for living organisms because it forms very stable complexes with transition metals that are essential for protein function [5]. Apart from acute toxicity that may result in death, consumption of sub-lethal doses of cyanide from cassava products over long periods of time, results in chronic cyanide toxicity that increases the prevalence of goiter and cretinism, *konzo* and tropical ataxic neuropathy [6, 7].

Linamarase (E.C. 3.2.1.21; linamarin–β–D–glucoside glucohydrolase) catalyses the hydrolysis of the cyanogenic glucoside, linamarin (2–(β-D-glucopyranosyloxy) isobutyronitrile) and a related compound lotaustralin (methyl linamarin) to release hydrocyanic acid (HCN) which is the toxic factor in cassava [8, 9]. Mkpong *et al.,* [10] and Ikediobi and Onyike [11], reported that the endogenous linamarase content of cassava could not permit the complete breakdown of linamarin. Many authors [12, 13] have suggested the inoculation of fermenting cassava with a linamarase-producing microorganism. It was demonstrated by Ikediobi and Onyike [11] that it is possible to reduce the garri toxicity by the addition of an exogenous linamarase during the fermentation. This study focuses on some properties and the purification of linamarase enzyme from *Lactobacillus delbrueckii* NRRL B-763.

Results and Discussion

The effect of particle size on the removal of cassava flour cyanide is shown in Figure 1. Cassava flour sample of 0.5 mm treated with crude linamarase enzyme showed a reduction from 2.1 mg HCN/10 g sample to 0.22 mg HCN/10 g sample after 40 h (89.5% reduction). Samples of 250 μm size showed a reduction from 2.1mg HCN/10 g sample to 0.16 mg HCN/10 g sample after 20 h (92.4% reduction). Samples of 150 μm size showed a reduction from 2.1 mg HCN/10g sample to 0.11 mg HCN/10 g sample after 20 h (95% reduction). Control sample showed a reduction from 2.1 mg HCN/10 g sample to 1.98 mg HCN/10 g sample after 40 h (5.7% reduction). The effect of particle size reduction on cassava cyanide removal

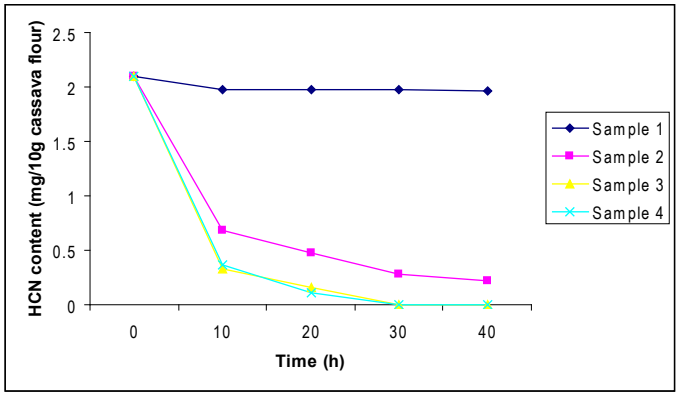

Figure 1. Detoxification of cassava flour samples by linamarase enzyme from *Lactobacillus delbrueckii* NRRL B-763. Sample 1, control; sample 2, cassava flour sample of approximately 0.5 mm in size; sample 3; cassava flour sample milled to pass 250 µm sieve; sample 4, cassava flour sample milled to pass 150 µm sieve.

was reported by Nambisan and Sundaresan [14] who observed that the efficiency of hydrolysis of cyanogenic glucoside of cassava tuber samples was increased when the samples were crushed. The linamarase enzyme of *Lactobacillus delbrueckii* NRRL B-763 is similar to the β-glucosidase from cassava [15] in cassava detoxification. The authors reported that addition of *Mucor circinelloides* crude linamarase enzyme during cassava tuber fermentation shortened and enhanced the detoxification process leading to a complete hydrolysis of cassava cyanogenic glucoside.

Table 1 shows the result of linamarase enzyme purification. The enzyme preparation obtained in step 4 of Table 1 represents about 40 fold purification with a recovery of about 33%. The elution pattern of the linamarase enzyme on Sephadex G-200 gel filteration chromatography is shown in Figure 2. From the result of Sephadex gel filteration chromatography, it was shown that the linamarase of *Lactobacillus delbrueckii* NRRL B-763 had an apparent molecular weight of 56 KDA. This result is similar to the findings of Esen (16), Mkpong *et al.,* [10] and Nok *et al.,* [17] who reported molecular weights of 60, 65 and 70 KDA respectively. Differences were found in the works of Eksittikul and Chulavatnatol [18] and Gueguen *et al.*, [19] who reported molecular weights of 600 KDA and 360 KDA respectively.

Effect of pH on activity and stability of the purified enzyme is shown in Figures 4 and 5. The enzyme had its optimum pH at 4.5. This finding is comparable to other β- glucosidases: Funaguma *et al.*, [20] reported an optimum pH of 4.5; Esen [16] showed an optimum pH of 5.8; Flores *et al.,* [21] and Gueguen *et al.,* (1997) reported an optimum pH of 5.5

The enzyme was stable under a pH range from 5.0 to 6.0 suggesting a possible utilization of this enzyme in detoxification process based on lactic acid fermentation [22, 23]. Some linamarases from lactic acid bacteria [24] as well as cassava β-glycosidases [18] did not show the same stability in acid pH.

The effect of temperature on activity and stability is shown in Figures 6 and 7. Maximum activity occurred at 50 ^{o}C and lowest activity at 0 ^{o}C. This finding is similar to Legras *et al.,* [25]; Flores *et al.,* [21], Esen [16] and Gueguen *et al.* [19] who reported temperature optima at 50 ^{o}C. Differences could be observed in Okafor and Ejiofor [24] who reported a temperature optimum at 29 + 2 ^{o}C. Nok *et al.* [17]) reported an optimum at 30 ^{o}C; Funaguma *et al.* [20] and Petruccioli *et al.* [15] reported optima at 60 ^{o}C.

Maximum temperature stability was observed between 40 and 50 ^{o}C. The enzymic activity gradually decreased with increasing temperature (Figure 7). A β-glucosidase from *Leuconostoc mesenteroides* exhibiting maximal stability between 40 and 50 ^{o}C has been reported [19].

Conclusion

From this work, it was concluded that the detoxification of cassava flour by crude linamarase enzyme was effected by particle size of the flour. The enzyme was purified to apparent homogeneity and showed both pH and temperature stability. Further studies and optimization studies could prove the linamarase enzyme useful for some industrial processes requiring cassava detoxification.

Table 1. Purification and recovery of linamarase enzyme of *Lactobacillus delbrueckii* NRRL B-763.

Purification step	Vol (mL)	Linamarase activity (U/mL)	Protein (mg)	Specific activity (U/mg)	% Yield	
Purification						Fold
Culture broth	250	226	71.4	3.17	100	1
$(NH_4)_2SO_4$	185	182	27.8	6.55	80.5	2.1
Precipitation (40%)						
$(NH_4)_2SO_4$	130	169	14.6	11.58	75	3.65
Precipitation (60%)						
Acetone precipitation	98	112	3.8	29.5	49.5	9.3
CMC column	210	89	0.86	103.5	39.4	32.65
Chromatography						

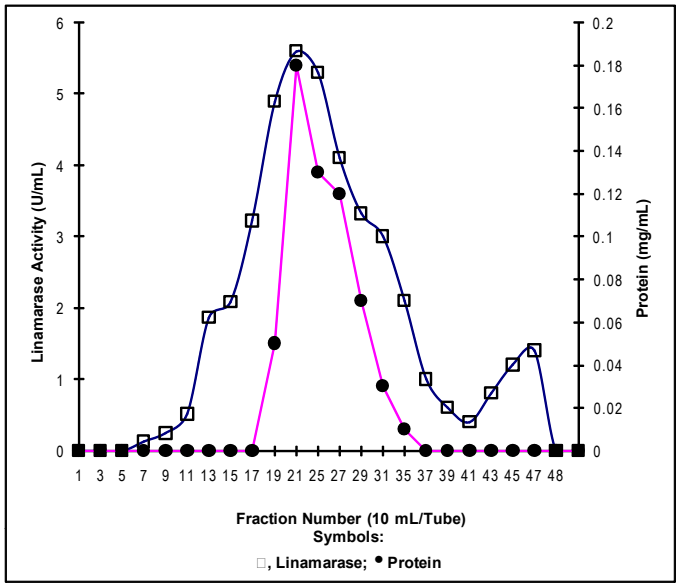

Figure 2. Sephadex G-200 column chromatography of the Linamarase enzyme of *Lactobacillus delbrueckii* NRRL B-763.

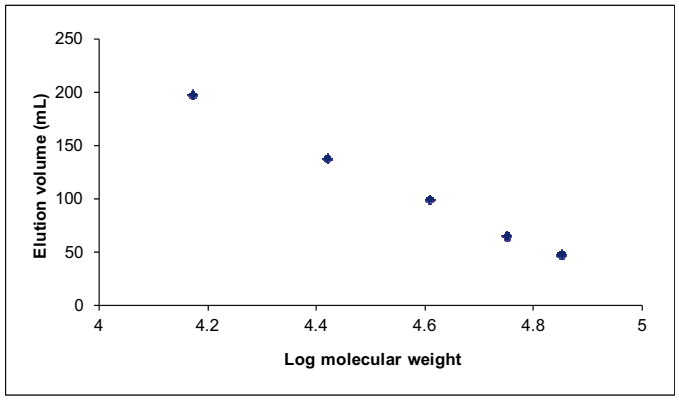

Figure 3. Estimation of molecular weight of linamarase enzme from *Lactobacillus delbrueckii* NRRL B-763 by Sephadex G-200 gel filteration chromatogrrarphy.

Experimental Section

Microorganism and enzyme isolation

Lactobacillus delbrueckii NRRL B-763 was kindly provided to the second author by Mr. James Swezey, ARS Culture and Patent Culture Collections Peoria, Illinois, USA. This organism was selected because of its high growth in a medium containing 800 parts per million KCN. Inoculum was prepared from a stock culture by transferring to an Erlenmeyer flask (250 mL) containing 100 mL of medium described by Okafor and Ejiofor (24): NaCl, 0.3%; $(NH_4)_2SO_4$, 0.1%; KH_2PO_4, 0.05%; $MgSO_4$, 0.02%; $CaCl_2$, 0.02%; lactose, 3%; linamarin (BDH, Poole England) 0.15%. The medium was sterilized at 121ºC for 15 minutes and adjusted to pH 6.5 with sterile lactic acid. The inoculum was grown for 24 h in a Gallenkamp orbital

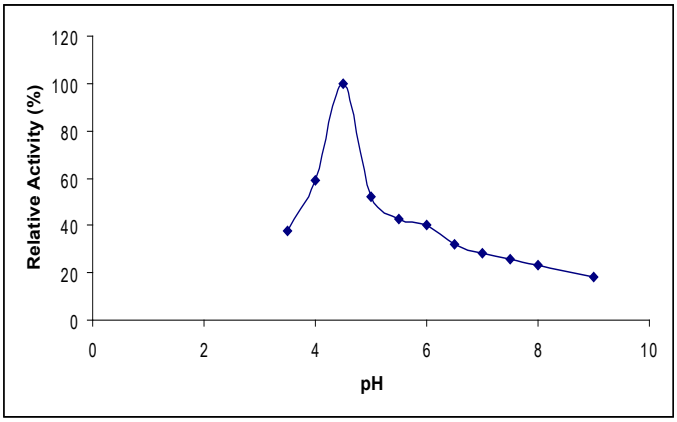

Figure 4. Effect of pH on enzyme activity.

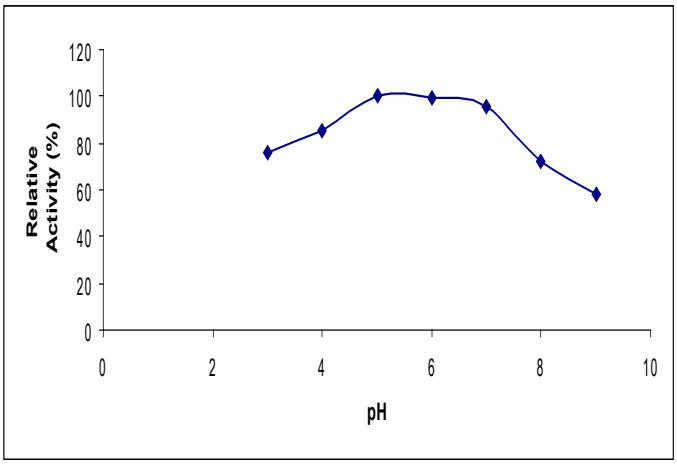

Figure 5. Effect of pH on enzyme stability.

incubator rotating at 100 × g at 30 ºC. Cells were harvested by centrifugation for 15 min using a Gallenkamp Junior centrifuge (No CF 405) with the speed setting at No 5. The supernatant fluid was chilled at 0 ºC. To release the cell-bound enzyme, cell suspension was disrupted by sonication (Biologics Ultrasonic homogenizer Model 150VT). Following disruption, the mixture was centrifuged at 2515 × g for 15 minutes and the supernatant was chilled to 0 ºC. The supernatants were combined and used for enzyme assay.

Assay procedures

Linamarase activity was assayed by determining the HCN librated from linamarin as follows: 0.5 mL of enzyme solution in 0.2M phosphate buffer (pH 6.5) contained in Eppendorf tubes was added to 0.5 mL of 1mM buffered (same buffer) solution of linamarin. After 20 minutes of incubation at 32 ± 2 ºC, 2 mL of 2% KOH and 1mL of picric acid: Na_2CO_3: H_2O (1:5:200 v/w/v) were added into the reaction mixture. The reaction was stopped by placing the tubes in iced water. The red colour that developed was read at 510 nm in a spectrophotometer. Under the above conditions, one unit of activity was defined as the

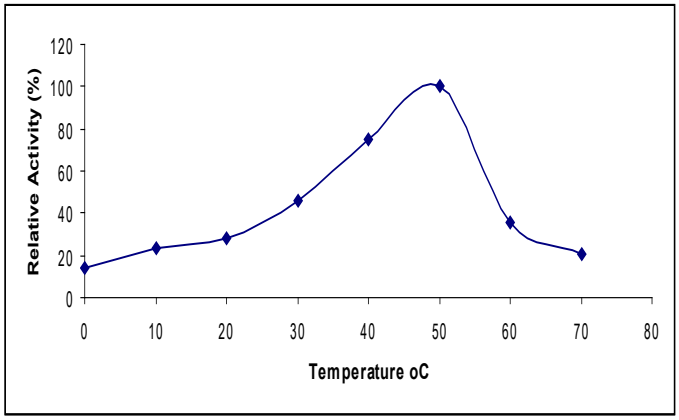

Figure 6. Effect of temperature on enzyme activity.

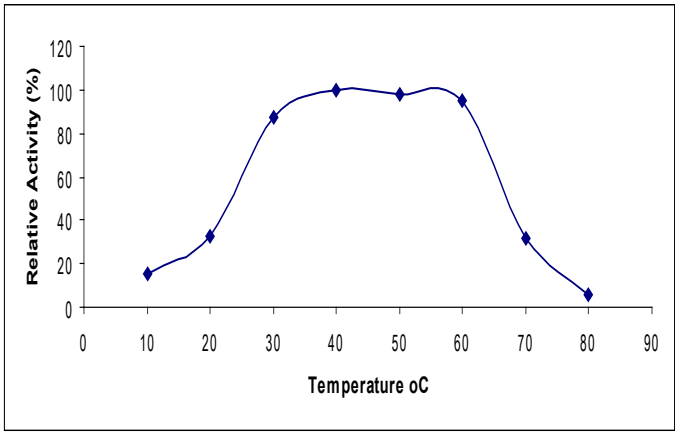

Figure 7. Effect of temperature on enzyme stability.

amount of enzyme that released 1µg HCN in 30 minutes under the assay condition.

The pH was determined using a glass electrode pH meter (PYE Unicam, England). Protein content was estimated by the method of Lowry *et al.*, [26] using bovine serum albumin (Sigma-Aldrich) as a standard. Cyanide was determined by a modification of the alkaline picric acid method of Williams and Edwards [27] as follows: various quantities of standard (50, 100, 150 and 200 µg/mL) solution of KCN were added into tubes containing 2 mL of 2% KOH and 1mL of picric acid: Na_2CO_3: H_2O (1:5:200 v/w/v). The tubes were incubated for 10 minutes in a 37 ºC water bath, cooled for 20 min in a refrigerator and read in a Spectrum lab 23A spectrophotometer at 510 nm. The readings were used to draw a standard curve for micrograms KCN per mL against absorbance.

Detoxification of cassava flour samples by crude linamarase enzyme from *Lactobacillus delbrueckii* NRRL B-763

Cassava tuber samples (NR 8082) were pretreated as follows: Sample 1, control, uninoculated cassava flour sample (*ca* 0.5 mm in size); sample 2, cassava flour sample of approximately 0.5 mm in size obtained after sieving ground samples; sample 3; cassava flour sample milled to pass 250 µm sieve; sample 4, cassava flour sample milled to pass 150 µm sieve. The samples (10 g each) contained in glass bottles were treated with 15 mL of buffered (0.2 M phosphate buffer, pH 6.5) crude linamarase enzyme 38 U/mL. Then 2 mL of 2% KOH and 1mL of picric acid: Na_2CO_3: H_2O (1:5:200 v/w/v) contained in test tube was suspended in each bottle just before the bottles were sealed and the system was incubated for 40 h at 32 ºC. Samples were examined at 10 h intervals. The HCN liberated from the cassava flour was absorbed by the alkaline picrate solution in the test tube [28]. After incubation, the reaction was stopped by placing the bottles in iced water. The color that developed was read at 510 nm. Cyanide levels were extrapolated from the KCN standard curve.

Enzyme purification and properties

Enzyme supernatant fluid was brought to 40% saturation with $(NH_4)_2$ SO_4 and was recovered by centrifugation at 2515 × g for 15 min and brought to 60% saturation by further addition of $(NH_4)_2$ SO_4. This was followed by centrifugation at 2515 × g for 15 minutes. Acetone (40% v/v) was added with further centrifugation. The supernatant was dialyzed overnight against 0.2 M phosphate buffer (pH 6.5) and applied to a carboxyl methyl cellulose column (7 × 62 cm) previously equilibrated with the same buffer and eluted with a linear NaCl gradient (0.1-0.8 M). Fractions (10 mL each) were collected and assayed for linamarase and protein content. Fractions showing linamarase activity were pooled and precipitated with cold acetone. The precipitate was collected by centrifugation and re-suspended in the same buffer. This suspension was subjected to gel filtration in a column of Sephadex G-200 (7 × 62 cm) pre-equilibrated with the same buffer.

Determination of enzyme approximate molecular weight

The molecular weight of the linamarase enzyme was determined by the method of Andrews [29] using Sephadex G- 200 column (7 × 62 cm) pre-equilibrated with phosphate buffer (pH 6.5). The molecular weight reference compounds were lysozyme (molecular weight, 14,000; BDH); trypsin (molecular weight, 23,000; BDH); egg albumin (molecular weight, 42, 000; BDH) and bovine serum albumin (molecular weight, 67,000;Sigma-Aldrich).

For the determination, 0.5 mg of each marker protein and 1mL of enzyme solution were added into 3 mL of 0.2M phosphate buffer (pH 6.5) and applied to a Sephadex G-200 column (7 × 62 cm) pre equilibrated with the same buffer. Elution was done at a flow rate of 25 mL/h and fractions (10 mL) were collected and assayed for linamarase activity and protein content. The enzyme elution volume was determined by estimating the activities of the fractions and proteins were determined by absorbance measurements at 280 nm. The approximated molecular weight of the enzyme was calculated from plots of elution volumes against logarithms of the molecular weights of the marker proteins.

The influence of pH on enzyme activity and stability

The effect of pH on activity of the linamarase enzyme was determined by using buffer solutions of different pH (acetate buffer, 3.5, 4.0, 4.5, 5.0 and 5.5); (phosphate buffer, 6.0, 6.5, 7.0 and 7.5) and (Tris-HCl buffer 8.0 to 9.0) for enzyme assay. The buffers were used at a concentration of 0.2 Mol/L. The pH activity profile of the linamarase enzyme was determined by incubating 0.5 mL of the enzyme contained in test tube with 0.5 mL of linamarin prepared in buffers of different pH values (3.5 to 7.5) at 50 ^{o}C for 1 h. The reaction was stopped by placing the tubes in iced water and the enzyme activities were determined. The percentage relative activities were calculated based on the pH values that gave the highest activity of the enzyme.

The pH stability profiles of the enzyme were determined by incubating 0.5 mL of the enzyme in buffers (pH 3.0- 9.0) for 3 h at 50 ^{o}C. At the end of the incubation, 0.5 mL of 1% (w/v) linamarin in 0.2 M phosphate buffer (pH 6.5) was added to the enzyme solution and the mixture further incubated at 50 ^{o}C for 1 h. The relative enzyme activity was determined.

The influence of temperature on enzyme activity and stability

The influence of temperature on enzyme activity was studied by incubating 0.5 ml of the enzyme solution contained in test tube and 0.5 mL of linamarin solution prepared in 0.2 M phosphate buffer (pH 6.5) for 30 min at various temperatures (0, 10, 20, 30 40, 50, 60 and 70 ^{o}C) in a thermostatic water bath. (Kottermann, Bremen, Germany). The reactions were stopped by placing the tubes in iced water. The enzyme activity was determined as previously described.

The thermal stabilities of the enzyme at 10 to 80 ^{o}C were determined by incubating the enzyme in test tubes at the various temperatures and withdrawing 0.5 mL of each enzyme after 30 min. The samples were chilled in ice and the residual activities determined by the addition of 0.5 mL of 1% (w/v) linamarin in 0.2 M phosphate buffer (pH 6.5) and incubating the mixture at 50 ^{o}C for 30 min. The reaction was stopped by placing the tubes in iced water and then the enzyme activities were determined.

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