ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 5 (1998) 343–347

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Stability of the enzyme (*S*)-hydroxynitrile lyase from *Hevea brasiliensis*

Michael Bauer [a,*], Roland Geyer [a], Matthias Boy [a], Herfried Griengl [b], Walter Steiner [a]

[a] *Institute of Biotechnology, SFB Biocatalysis, Technical University Graz, Petersgasse 12, 8010 Graz, Austria*
[b] *Institute of Organic Chemistry, SFB Biocatalysis, Technical University Graz, Stremayrgasse 16, 8010 Graz, Austria*

Received 26 September 1997; accepted 26 November 1997

Abstract

Inactivation of the enzyme (*S*)-hydroxynitrile lyase from *Hevea brasiliensis* (rubber tree) was studied using three different buffers: sodium glutamate, sodium phosphate and sodium citrate. Inactivation follows a first order kinetics and was expressed by the half-life of the enzyme. From the inactivation at different temperatures, the inactivation energy was calculated for the different buffer systems; no significant difference could be found. Stability decreases rapidly with lower pH values in all three buffer systems and the enzyme is very unstable at pH 3.5. At pH 6.5 half-life increases with higher buffer concentrations in all three buffers whereas at pH 3.5 half-life decreases with higher buffer concentrations. Stabilisers improved the stability of the enzyme with increasing concentrations.

Keywords: Hydroxynitrile lyase; Enzyme stability

1. Introduction

In their natural environment hydroxynitrile lyases (EC 4.1.2.10) from plants catalyse the cleavage of cyanohydrins from cyanogenic glycosides into the corresponding aldehydes or ketones and HCN [1,2]. In the reverse reaction hydroxynitrile lyases can be used for the synthesis of enantiomerically pure cyanohydrins [3,4]. Cyanohydrins can be converted into a wide range of chiral compounds which are widely used for fine chemicals, pharmaceuticals and agrochemicals. Therefore hydroxynitrile lyases have become of growing industrial interest in the past decade.

Cyanohydrins are formed from aldehydes or ketones and HCN by pure chemical reaction without enzyme in aqueous systems at higher pH value [5]. This chemical reaction is responsible for the decrease of the enantiomeric excess of the cyanohydrin in an enzymatic process. On the contrary, hydroxynitrile lyase loses its activity below pH 5 very quickly [6]. Therefore it is necessary for organic synthesis to find the optimal reaction conditions where the enzyme is

* Corresponding author. Tel.: +43-316-873-8408; fax: +43-316-873-8434; E-mail: bauer@biote.tu-graz.ac.at

1381-1177/98/$19.00
PII: S1381-1177(98)00030-7

relatively stable and the chemical reaction can be mostly suppressed. Consequently detailed investigations on enzyme stability are necessary. The influence of different buffer systems, buffer concentrations, pH and different stabilisers was studied.

2. Materials and methods

2.1. Chemicals and enzyme

Purified racemic mandelonitrile was a gift from DSM-Chemie Linz and contained less than 0.5% benzaldehyde. All other chemicals were of p.a. quality.

Purified (*S*)-hydroxynitrile lyase from *Hevea brasiliensis* was provided by the Genetic Engineering Group, Department of Biotechnology, Technical University Graz. The enzyme was expressed in *Pichia pastoris* and purified by ion exchange chromatography [7–9].

2.2. Methods

2.2.1. Enzyme activity

Enzyme activity was measured following the cleavage of racemic mandelonitrile into benzaldehyde and HCN at a substrate concentration of 6 mM and at an enzyme concentration of 0.74 μg/ml. The assay was performed in 20 mM glutamate buffer at pH 5 and 25°C and the formation of benzaldehyde was monitored spectrophotometrically at 280 nm. Besides the enzymatic reaction a chemical side reaction takes place which has to be measured separately. The reaction was monitored for 2 min. From the slope ΔE vs. time enzyme activity is calculated. For all measurements duplicates were made.

In all inactivation experiments samples were taken at distinct intervals and enzyme activity was determined. The activity was followed at least until half-life was reached.

2.2.2. Influence of temperature

To determine the influence of temperature, the enzyme solution was incubated at 30, 40, 50, 60 and 70°C in 5 mM buffer solutions of glutamate, phosphate and citrate at pH 6.5.

2.2.3. Influence of pH

The enzyme was incubated at pH 3.5, 5.0 and 6.5 in 5 mM buffer solutions of glutamate, phosphate and citrate at 30°C.

2.2.4. Influence of buffer concentration

To investigate the influence of buffer concentration, the enzyme was incubated in the three buffer systems mentioned above at concentrations of 5, 20, 50 and 100 mM at pH 6.5, 40°C, and at pH 3.5, 40°C.

2.2.5. Influence of stabilisers

To improve the stability of the enzyme at low pH, different additives were added to the enzyme solution (5 mM glutamate buffer, pH 3.5, 30°C). Sorbit, sucrose, lactose and glycerine were tested and varied between 50 and 400 mg/ml.

2.2.6. Stability in glutamate buffer

The enzyme solution in 5 mM glutamate buffer was incubated at 30°C at different pH: 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0. Activity was monitored for 1 h.

3. Results and discussion

Inactivation of the hydroxynitrile lyase can be described by a first order kinetics. The inac-

Table 1
Half-life of hydroxynitrile lyase in different buffers in dependence on temperature

Buffer [5 mM]	Half-life [min]				
	30°C	40°C	50°C	60°C	70°C
Phosphate	1755	690	342	56	10
Glutamate	1379	204	80	42	15
Citrate	2315	322	115	29	7

Table 2
Inactivation energy for hydroxynitrile lyase in 5 mM buffer

Buffer	$E_{a,inact}$ [kJ/mole]
Phosphate	93
Glutamate	96
Citrate	110

tivation constant k_d was determined by non linear least squares fitting of the measured activities to a first order kinetics. Good correspondence between the measured values and the simulated equation could be achieved. Half-life was calculated from the inactivation constant k_d.

3.1. Influence of temperature

Half-life of the enzyme decreases with temperature in all three buffer systems (Table 1). The inactivation energy $E_{a,inact}$ can be calculated according to the law of Arrhenius [10].

$$k_d = A \exp\left(\frac{E_{a,inact}}{RT}\right)$$

At pH 6.5 the enzyme is stable for hours at 30°C whereas above 70°C the enzyme is inactivated rapidly (Table 1). The inactivation energy calculated from the inactivation constants at different temperatures does not differ significantly between the buffer systems (Table 2).

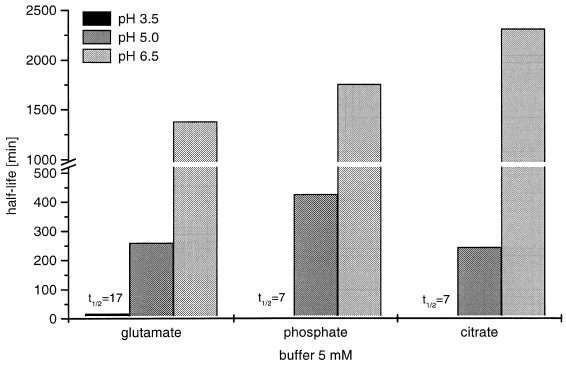

Fig. 1. Influence of pH on enzyme stability at 30°C.

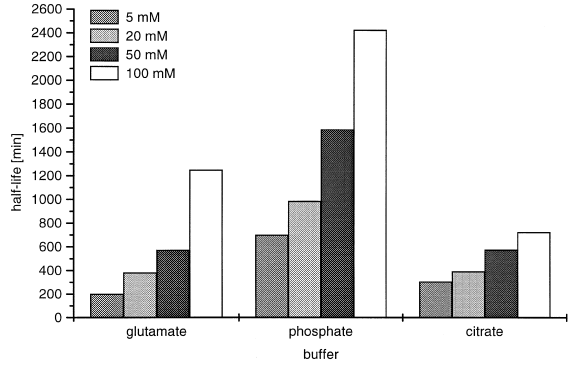

Fig. 2. Influence of buffer concentration on enzyme stability at 40°C, pH 6.5.

Inactivation energies around 100 kJ/mole are common for hydrolytic enzymes [11].

3.2. Influence of pH

Above pH 6.5 where the enzyme is very stable, it is nearly impossible to measure activity due to the fast chemical side reaction in the activity assay. Activity decreases with pH and at pH 3.5, the enzyme is inactivated very fast in all three buffer systems. This fast inactivation for the *H. brasiliensis* enzyme at low pH has been reported before [6,12]. Enzyme inactivation at extreme pH is generally due to irreversible ionisations which lead to the unfolding of the protein [13]. Glutamate buffer seems to

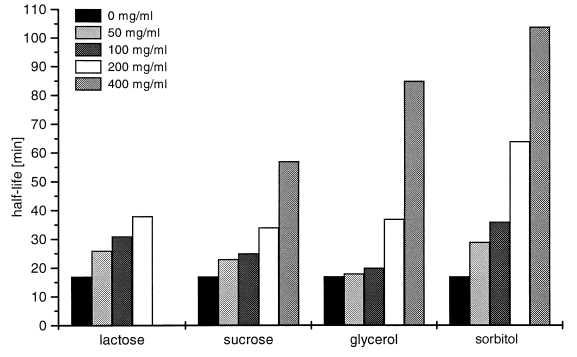

Fig. 3. Influence of additives on enzyme stability in 5 mM glutamate buffer, pH 3.5, 40°C.

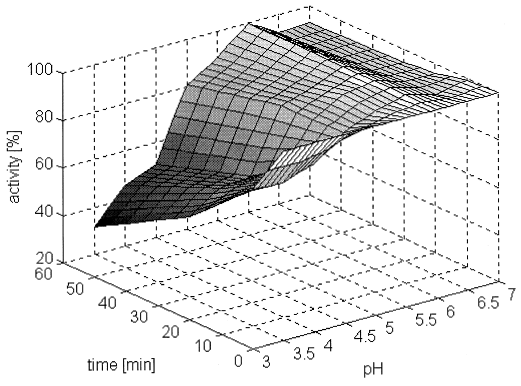

Fig. 4. Enzyme stability in 20 mM glutamate buffer, 30°C.

stabilise the enzyme best at low pH as the half-life is twice in comparison to citrate and phosphate (Fig. 1). The explanation might be that glutamate itself is not only a buffer substance but also an amino acid which interacts with the enzyme.

3.3. Influence of buffer concentration

At pH 6.5 stability increases with increasing buffer concentration in all three systems (Fig. 2). The enzyme is most stable in phosphate buffer whereas for glutamate buffer the relative increase of half-life with concentration is more pronounced. In contrast to these results stability decreases with higher buffer concentrations at pH 3.5 in all three buffer systems (data not shown) which corresponds to previous investigations [6,12]. The inverse effect at high pH cannot be explained yet properly. Maybe different charge distributions account for this phenomenon.

3.4. Influence of stabilisers

Stability could be improved with all four tested additives with increasing concentrations (Fig. 3). Sorbit improves stability even six times but only at high concentrations (400 mg/ml). With all other additives, a maximal three fold increase in half-life could be achieved. Improved enzyme stabilisation by sugars and polyols has been frequently reported assuming that the addition of these additives strengthens the hydrophobic interactions among non polar amino acid residues making them more resistant against unfolding [6,14,15].

3.5. Stability in glutamate buffer

Detailed investigations were performed in glutamate buffer as this buffer was used for further kinetic studies. Above pH 6.0 the enzyme does not lose activity within 1 h. Below pH 5 activity decreases dramatically and at pH 3.5 only about 30% activity are left after 1 h (Fig. 4). Consequently a pH of 5 or higher should be chosen if it is necessary to retain activity over a long period of time.

4. Conclusion

To obtain a high enantiomeric excess of the cyanohydrin in aqueous systems a low pH is necessary. Under these conditions one should work at low temperatures and at low buffer concentrations in combination with stabilisers in order to increase enzyme stability.

References

[1] A. Hickel, M. Hasslacher, H. Griengl, Physiol. Plant. 98 (4) (1996) 891–898.
[2] H. Wajant, F. Effenberger, Biol. Chem. 377 (10) (1996) 611–617.
[3] M. Schmidt, S. Herve, N. Klempier, H. Griengl, Tetrahedron 52 (23) (1996) 7833–7840.
[4] F. Effenberger, Enantiomer 1 (1996) 359–363.
[5] D. Selmar, F.J.P. Carvalho, E.E. Conn, Anal. Biochem. 166 (1987) 208–211.
[6] A. Hickel, M. Graupner, D. Lehner, A. Hermetter, O. Glatter, H. Griengl, Enzyme Microb. Tech., in press.
[7] M. Hasslacher, M. Schall, M. Hayn, H. Griengl, S.D. Kohlwein, H. Schwab, J. Biol. Chem. 271 (10) (1996) 5884–5891.
[8] M. Hasslacher, M. Schall, M. Hayn, H. Griengl, S.D. Kohlwein, H. Schwab, Ann. N.Y. Acad. Sci. 799 (1996) 707–712.

[9] H. Wajant, S. Förster, Plant Sci. 115 (1996) 25–31.
[10] M. Dixon, E.C. Webb, Enzymes, Longman, London, 1979, p. 171.
[11] H. Netter, Theoretical Biochemistry, Oliver and Boyd, Edinburgh, 1969, p. 656.
[12] M. Schall, Ph.D. Thesis, University Graz, 1996.
[13] K.F. Tipton, H.B.F. Dixon, Method. Enzymol. 63 (1979) 183–234.
[14] A.M. Klibanov, Adv. Appl. Microbiol. 29 (1983) 1–28.
[15] C.J. Gray, Biocatalysis 1 (1988) 187–196.