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Influence of Flavonoids on the Stability of an (*S*)-Hydroxynitrile Lyase from *Hevea brasiliensis*

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Dedicated to the memory of Professor Vera Johanides

Summary

The (*S*)-hydroxynitrile lyase from leaves of the rubber tree *Hevea brasiliensis* (*Hb*Hnl) (EC 4.1.2.39) catalyzes the industrially interesting formation of (*S*)-cyanohydrins from aldehydes or ketones and HCN. The overall yield of the (*S*)-cyanohydrin is reduced by the non-stereoselective base catalyzed chemical background reaction. To reduce the chemical background reaction, lower pH values (below pH=5) are necessary, but the enzyme in this case is not stable enough. To stabilize the enzyme at these conditions the addition of distinct effectors might be helpful. The starting point of the stabilizing experiments was the extract of rubber tree leafs. Therefore the leafs were extracted with potassium citrate/phosphate buffer and this extract then chromatographically separated into 5 fractions. The total extract and the fractions thereof were tested for their effect on the enzyme half-life. The extract improved the half-life up to 5 times and one chromatographic fraction increased the stability by 50 %. This fraction was further analyzed and it was found that flavonoids were present. In the next step some pure flavonoids were tested for their effect and it was found that low concentrations of rutin (5–20 ng/mL) and hyperoside (1.5–6 ng/mL) were sufficient to improve enzyme stability up to 50 %. In addition, some synthetic flavonoids (Venoruton® and monohydroxyethylrutoside) were tested and they also prove this stabilizing effect. The increase of half-life was even higher, almost fivefold in optimal conditions. The increase in the stability is assigned to a chelating effect on the one hand and a structural effect on the other hand. The impact of these effects cannot be presently differentiated.

Key words: hydroxynitrile lyase, *Hevea brasiliensis*, stabilization, flavonoids

Introduction

The natural function of hydroxynitrile lyases (Hnls) is the cleavage of cyanogenic glycosides into the corresponding aldehydes or ketones and HCN *(1)*. The reverse reaction of the Hnls can be used to synthesize

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enantiomerically pure cyanohydrins *(2–7)*. These cyanohydrins may be converted into a wide range of chiral compounds for their use as fine chemicals, pharmaceuticals or agrochemicals.

The problem during the enzymatic synthesis is the chemical background reaction, where the addition of HCN to the aldehyde or ketone leads to a racemat of the cyanohydrin, which concomitantly reduces the enantiomeric excess *(8)*. To avoid the base catalyzed chemical reaction, the pH value for the enzymatic synthesis is shifted into the acidic region. Under these conditions the stability of the enzyme is significantly reduced at pH values below pH=5 *(9,10)*. Therefore the reaction conditions have to be optimized for a maximum stability and reactivity of the enzyme resulting in high ee-values. Possibilities to overcome these situations are immobilization, working in a two-phase system, adding stabilizing substances or combinations of these methods *(11–16)*.

For many enzymes it is known that they are relatively stable in their natural environment, but they are quite unstable when they are purified. So we tried to look for some stabilizing substances occurring in the natural environment of the enzyme *(17,18)*.

The original source of the used hydroxynitrile lyase are the leaves of the rubber tree *Hevea brasiliensis* from where the enzyme was isolated and then overexpressed in *Pichia pastoris.* The comparison of the natural enzyme with the in *Pichia pastoris* overexpressed enzyme showed differences in their stability. It indicated that some compounds of the plant leaves were responsible for higher stability. The leaves were therefore cut up and extracted. The extract and the fractions of it were used as additives. At the end flavonoids were found as part of one of these fractions and pure forms were added for more detailed investigations.

Whilst a great number of publications deal with the inhibition and stimulation of enzymes by flavonoids no reports could be found particularly on enzyme protection by flavonoids *(19–21)*. This was the starting point for the present study.

Materials and Methods

Enzyme and chemicals

The recombinant *Hb*Hnl solution was provided by Roche Molecular Biochemicals (Penzberg, Germany) with a protein concentration of 62 mg/mL and a specific activity of 96 IU/mg of protein (standard activity test, mandelonitrile as substrate). The enzyme was overexpressed in *Pichia pastoris* and about 80 % of the total protein concentration in this preparation was *Hb*Hnl. For all measurements the enzyme concentration was 7.7 μg/mL.

Purified racemic mandelonitrile was a gift from DSM-Chemie Linz (Linz, Austria).

Quercetin, hyperoside, isoquercitrin, rutin, kaempherol-3-rutoside and kaempherol-7-neohesperidoside were purchased from Roth (Karlsruhe, Germany).

Monohydroxyethylrutin and Venoruton® were a gift from Gebro (Fieberbrunn, Austria).

All other chemicals were of p.a. quality.

Preparation of an extract from Hevea brasiliensis leaves

A mass of 42 g of leaves from a rubber tree (a gift from the Botanic Garden Graz, Graz, Austria) were first cut into small pieces by a scissors and then homogenized for two hours with an Ultraturax at 9500 min^{-1} and 4 °C. After adding 150 mL potassium citrate/phosphate buffer (pH=5.0, 20 mM) the resulting paste was further mixed with the Ultraturax at 9500 min^{-1} for additional two hours. For the final extraction the homogenate was vigorously stirred for another two hours at 4 °C on a magnetic stirrer. The mixture was frozen at –18 °C and then after defrosting and resuspension centrifuged for 1 hour at 10 000 rpm and 4 °C. The turbid supernatant was filtered through a glass microfibre filter Whatman GF/A (Maidstone, England) and the clear slightly yellow filtrate was subsequently lyophilized. The resulting powder was stored and used as »extract« in this study.

*Isolation of flavonoids from the crude enzyme Hb*Hnl

A mass of 10 g crude *Hb*Hnl from rubber tree leaves (a gift from the Rubber Research Institute of Malaysia, Kuala Lumpur, Malaysia) were extracted with 600 mL methanol under a reflux condenser on a magnetic stirrer for five hours at room temperature. After decanting the methanol, the extraction was repeated with 500 mL methanol for three hours. The combined methanolic extracts were evaporated at room temperature and the residue dissolved in sufficient methanol to obtain a clear solution (approx. 100 mL). The solution was subjected to size exclusion chromatography on Sephadex LH 20 using methanol as eluent. Thin-layer chromatography (TLC) was used to follow the chromatographic procedure (TLC system see below). After a first run, 12 fractions of 25 mL each were collected. Based on the TLC data these 12 fractions were pooled to five major fractions (I to V) and tested for their effect on *Hb*Hnl.

From these 5 methanolic fractions the methanol was evaporated getting rid of its deactivating effect on the enzyme. The residues from this evaporation were suspended in 4 mL potassium citrate/phosphate buffer pH=4.0, 20 mM and stirred for 30 min at 25 °C. After this extraction the suspension was centrifuged for 15 min at 4000 min^{-1} and 4 °C. The supernatants were adjusted to pH=4.0 and taken as additives in different volumes.

Fraction II caused an *Hb*Hnl enhancing effect. After TLC and HPLC analysis, this fraction could be proven as flavonoid containing.

Thin-layer chromatography (TLC)

Silicagel plate 60 F254 (Merck 5554)

Mobile phase:	EtOAc/EtCOMe/HCOOH/H_2O (5/3/1/1)
Detection:	Diphenylboric acid 2-aminoethyl ester (1 % in methanol) PEG 4000, UV detector at 366 nm

High-pressure liquid chromatography (HPLC)

Apparatus:	Merck/Hitachi L 6200 Intelligent Pump L 4500 Photo diode Array Detector

Stationary phase:	Pre column: LiChrospher 100 RP-18 (Merck) Column: LiChrospher 100 RP-18, 250 × 4 mm (Merck)
Mobile phase:	AcCN/H_2O (+ 1 % H_3PO_4) 17/83
Flow:	1 mL/min, isocratic
Detection:	254 nm
Injection:	20 μL
Run time:	40 min

The main flavonoids were isolated by preparative TLC (TLC system see above). Under these conditions approx. 80 mg of kaempferol derivative (P2) and approx. 190 mg of a mixture of rutin and kaempferol-3-rutinoside (DP) could be obtained.

Used flavonoids

Because the fraction containing flavonoids gave a positive effect on the half-life, further studies on the enzyme stability were performed using following pure flavonoids (Fig. 1).

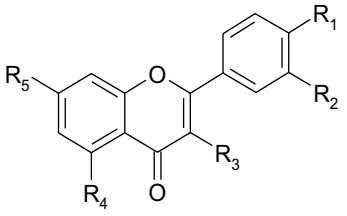

Fig. 1. Basic structure of the flavonoids

The selection of the flavonoids was carried out according to the chromatographic results and literature *(22)*.

For the investigations 6 pure flavonoids (Q, H, I, R K3 and K7, for description see Table 1) isolated from natural sources and 2 synthetic forms (M and V) were chosen.

Activity test and stability studies

The standard activity test was performed as described earlier *(23)* with the difference of using potassium citrate/phosphate buffer instead of glutamate buffer *(24)*. The wavelength for the measurement of the chromatographically separated fractions was shifted to 300 nm because of the high self-absorption of the substances.

Incubation conditions for the stability studies:

Fractions I-V: potassium citrate/phosphate buffer 20 mM, pH=4.0 and 25 °C

Flavonoids: potassium citrate/phosphate buffer 20 mM, pH=4.25 and 25 °C

For the resulting solutions the pH values were adjusted if necessary. The enzyme was diluted with these solutions to a concentration of 7.7 μg/mL and incubated at 25 °C at least until the half-life time of the enzyme was reached.

Study of the influence of oxygen on the enzyme

The enzyme was tested for a direct deactivating effect of the dissolved oxygen in the buffer. Therefore the buffer for the enzyme incubation, potassium citrate/phosphate buffer 20 mM, pH=4.25 and 25 °C was on the one hand degassed by a vacuum pump (buffer was evacuated for 1 h with an membrane pump) and on the other hand saturated with oxygen by gassing the buffer with air. The air was finely distributed by using a glass frit under atmospheric pressure in a gas washing bottle for 60 min.

In these buffer solutions the enzyme was incubated at 25 °C at least until the enzyme half-life time was reached.

Study of the influence of EDTA on the enzyme

As in literature flavonoids were somehow related with chelating capacity *(25–27)* a »synthetic« chelator was tested for his effect on the enzyme. EDTA was deliberately chosen for these investigations.

Table 1. Studied pure flavonoids (short forms in brackets)

	R_1	R_2	R_3	R_4	R_5
Quercetin (Q)	OH	OH	OH	OH	OH
Hyperoside (H)	OH	OH	O-β-D-galactopyranosyl	OH	OH
Isoquercitrin (I)	OH	OH	O-β-D-glucopyranosyl	OH	OH
Rutin (R)	OH	OH	O-[6-O-(6-deoxy-α-α-mannopyranosyl)-β-D-glucopyranosyl]	OH	OH
Kaempherol-3-rutoside (K3)	OH	H	O-[6-O-(6-deoxy-α-α-mannopyranosyl)-β-D-glucopyranosyl]	OH	OH
Kaempherol-7-neohesperidoside (K7)	OH	H	OH	OH	O-[2-O-(6-deoxy-α-α-mannopyranosyl)-β-D-glucopyranosyl]
Monohydroxylethylrutin (M)	$O(CH_2)_2OH$	OH	O-[6-O-(6-deoxy-α-α-mannopyranosyl)-β-D-glucopyranosyl]	OH	OH
Venoruton® (V)	$O(CH_2)_2OH$	OH (85 %), $O(CH_2)_2OH$ (15 %)	O-[6-O-(6-deoxy-α-α-mannopyranosyl)-β-D-glucopyranosyl]	OH (40 %), $O(CH_2)_2OH$ (60 %)	$O(CH_2)_2OH$

An EDTA standard solution was prepared in potassium citrate/phosphate buffer 20 mM, pH = 4.25 and 25 °C. In these so prepared buffer solutions (5.1 – 0.0017 ng/mL) the enzyme was incubated at 25 °C at least until the enzyme half-life time was reached.

Results and Discussion

Stabilization using leaf extract

The values for the extract concentrations were corrected by subtracting the amount of buffer salts, which were introduced during the extraction procedure from the leaves.

The additional buffer salts in the extract powder do not affect the enzyme half-life. This was proven in a blank by incubating the enzyme in a 40 mM potassium citrate/phosphate buffer solution without extract and determination of the enzyme half-life. The determined values of 38 and 40 min in a 40 mM buffer did not vary from the values in the 20 mM buffer (39 and 40 min).

Fig. 2 clearly shows the positive effect of the leaf extract on the enzyme stability up to 6 mg extract·mL^{-1}. The half-life increases fivefold, from 39 min (without extract) to the maximum of 195 min at 6 mg/mL and then decreases with higher extract concentrations. This might be explained by an inhibition effect, because with the increasing extract concentration the initial activity of the enzyme also drops (see Fig. 3 a,b).

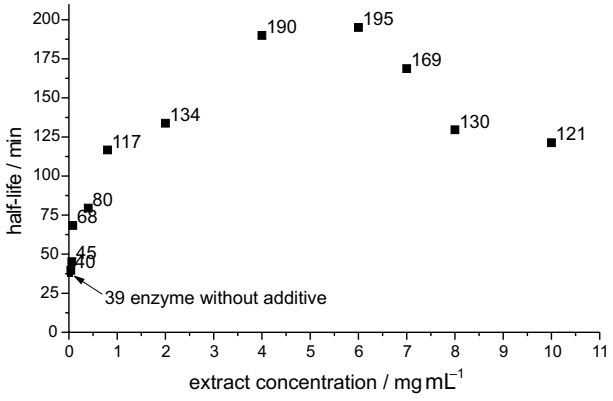

Fig. 2. Influence of different leaf extract concentrations on the enzyme half-life Conditions: potassium citrate/phosphate buffer, pH=4.25, 20 mM and 25 °C

With increasing concentrations of the leaf extract the initial activity of the enzyme drops. This drop is accompanied by a flattening of the activity time course. These flat curves lead to a relatively high half-life in the extract concentration range from 0.08 to 4 mg/mL. At higher concentrations (>4 mg·mL^{-1}) the initial activity is 40 to 50 % less than the starting activity of the enzyme without extract. The exact reason for this phenomenon is not yet known; maybe some inhibition or destabilization takes place.

Stabilization using fractions of the extract

For this study only the buffer soluble substances of the chromatographic fractions I-V were taken (Table 2). Because of its deactivating effect methanol was replaced by buffer (see Isolation of flavonoids from the crude enzyme *Hb*Hnl).

Table 2. Concentrations and extraction sequence of the individual 5 fractions (I-V)

	I	II	III	IV	V
	γ/mg mL^{-1}				
Methanol soluble	26.4	10.5	3.4	15.9	13.8
Buffer insoluble	5.3	2.2	0.3	0.5	0.1
Buffer soluble	21.1	8.3	3.1	15.4	13.7

Conditions: potassium citrate/phosphate buffer, pH=4.0, 20 mM and 25 °C

The five buffer fractions were tested for their effects on the enzyme stability. The finally used fractions are marked in the gray shaded line (Table 2). In order to study the influence of the individual fractions on the half-life of the *Hb*Hnl enzyme, different concentrations were applied (Table 3).

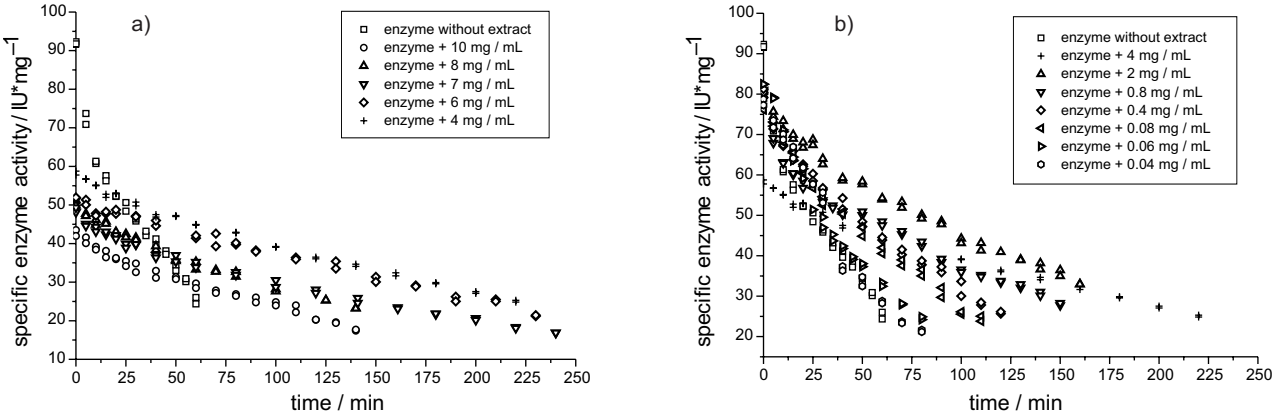

Fig. 3. The activity time course with varying leaf extract concentrations; a) 4–10 mg/mL, b) 0.04–4 mg/mL
Conditions: potassium citrate-/phosphate buffer, pH=4.25, 20 mM and 25 °C

Table 3. The influence of different concentrations of fractions I – V on the enzyme half-life

γ(I)	$t_{½}$	γ(II)	$t_{½}$	γ(III)	$t_{½}$	γ(IV)	$t_{½}$	γ(V)	$t_{½}$
mg mL^{-1}	min	mg mL^{-1}	min	mg mL^{-1}	min	mg mL^{-1}	min	mg mL^{-1}	min
6.34	15	1.66	53	0.92	27	4.62	34	4.11	25
4.22	17	1.11	45	0.61	28	3.08	35	1.82	27
2.11	25	0.83	42	0.31	28	1.54	31	1.37	27
1.06	31	0.42	40	0.15	26	0.77	32	0.68	30
0.53	33	0.21	35	0.08	30	0.38	36	0.34	32

Conditions: potassium citrate/phosphate buffer, pH=4.0, 20 mM and 25 °C (enzyme without additive $t_{½}$= 32 min)

The results from these investigations showed clearly that only fraction II had a reasonable positive effect on the enzyme stability. The half-life increased up to 60 % with the highest added concentration. Fraction I seems to contain compounds with inhibiting or destabilizing properties, and was therefore not considered for further investigation.

As previously mentioned fraction II contains flavonoids (see Material and Methods). Therefore some pure flavonoids (I, R, Q, K3, K7 and H) were tested, as well as the fractions (P2, DP) isolated by preparative TLC. Two half synthetic flavonoids (M and V) were also tested. The results are given in Table 4.

Of all pure flavonoids studied, only rutin and hyperoside showed a stabilizing effect on the enzyme by increasing the half-life up to 50 – 60 %. One problem of these substances is their low solubility in the buffer system. The highest given concentrations in Table 4 are around the maximum solubility of the used substances and could not be increased.

Higher concentrations could be only reached (see Table 5) with some synthetic products (M and V) and the isolates from preparative TLC (DP and P2; see Isolation of flavonoids from the crude enzyme *Hb*Hnl).

These substances have a much higher solubility compared to the tested pure flavonoids. The upper limit of the concentration in this study is given by the self-absorption of the substances under the assay conditions.

But it is clear from the results that there is a pronounced stabilizing effect from these substances causing an increase of the half-life up to 4 fold. Comparing this data with data already available for the stabilization of the enzyme with sugars *(9,10)* clearly shows that the flavonoids give a positive effect at much lower concentrations. While 50 to 100 mg/mL polyols are necessary

Table 4. The effect of different concentrations of pure flavonoids on the enzyme half-life

γ(**I**)	$t_{½}$	γ(**R**)	$t_{½}$	γ(**Q**)	$t_{½}$	γ(**K3**)	$t_{½}$	γ(**K7**)	$t_{½}$	γ(**H**)	$t_{½}$
mg mL^{-1}	min	mg mL^{-1}	min	mg mL^{-1}	min	mg mL^{-1}	min	mg mL^{-1}	min	mg mL^{-1}	min
0.03	44	0.02	79	0.01	44	0.005	46	0.005	46	0.006	67
0.015	42	0.015	72	0.005	42	0.0025	44	0.0025	41	0.0045	57
		0.01	65							0.003	54
		0.005	53							0.0015	47

Conditions: potassium citrate/phosphate buffer, pH=4.25, 20 mM and 25 °C (enzyme without additive $t_{½}$= 44 min)

Table 5. Different concentrations of synthetic flavonoids and TLC-fractions and their influence on the enzyme half-life

γ(**DP**)	$t_{½}$	γ(**P2**)	$t_{½}$	γ(**M**)	$t_{½}$	γ(**V**)	$t_{½}$
mg mL^{-1}	min	mg mL^{-1}	min	mg mL^{-1}	min	mg mL^{-1}	min
0.62	254	–	–	0.5	119	0.5	172
0.3	199	0.3	178	0.25	71	0.4	149
0.1	152	0.1	108	0.1	59	0.2	121
0.05	120	0.05	91	0.05	54	0.1	95
0.01	70	0.01	55	0.01	46	0.075	72
0.005	58	0.005	47	–	–	0.05	69
–	–	–	–	–	–	0.01	65

Conditions: potassium citrate/phosphate buffer, pH=4.25, 20 mM and 25 °C (enzyme without additive $t_{½}$=44 min)

to reach a reasonable positive effect, the necessary amount of flavonoids is at least a 100 times less.

The mechanism behind is not yet clear. It is reported that flavonoids may act as antioxidants, chelating agents or radical scavengers *(25–27)*. The subsequent investigations should clarify how the flavonoids influence the enzyme stability.

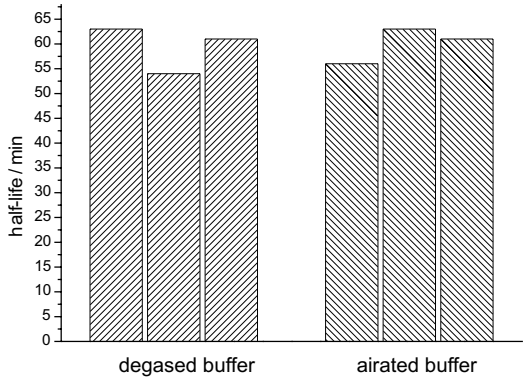

Fig. 4. Oxygen influence on the enzyme stability
Conditions: potassium citrate/phosphate buffer, pH=4.25, 20 mM and 25 °C

Influence of oxygen on the enzyme stability

From the tested, degassed and air saturated buffer systems no significant effect of the oxygen concentration could be observed.

There was no direct effect of the oxygen concentration on the enzyme half-life. If oxygen would influence the enzyme stability to certain extent only oxygen radicals, so called reactive oxygen species produced by some metal ions in the system could be responsible for such an effect. Therefore we tried to reduce the concentration of potential radical-producing transition metal ions like Fe^{2+}, Cu^{2+}, Zn^{2+} or Mg^{2+}, and the concentration of the radicals itself *via* chelating *(25–27)*. In order to complex these ions and/or radicals the effect of EDTA on the enzyme was studied.

Effect of EDTA on the enzyme

From these experiments a clear effect on the half-life was observed (Table 6).

Table 6. Influence of different EDTA concentrations on the enzyme half-life

γ(**EDTA**)/ng mL^{-1}	$t_{1/2}$/min
0	55
17	31
5.1	54
1.7	78
0.17	77
0.017	74
0.0017	66

Conditions: potassium citrate/phosphate buffer, pH=4.25, 20 mM and 25 °C

With high concentrations of EDTA (5 – 17 ng/mL) there is a deactivating effect from EDTA itself. Lowering the EDTA concentration (below 5 ng/mL) the chelating action of EDTA seems to become the major contribution, leading to an increase of the half-life up to 50 %.

The prolongation of the enzyme half-life by adding EDTA could be related to a reduction of the radical forming agents (*27*). For the flavonoids an additional effect of chelating reactive oxygen species was reported in literature (*26,27*). The extent of the increase in stability by the flavonoids cannot be explained by these factors alone. An additional structural effect is assumed, which also could improve the enzyme stability but it is not clearly understood yet.

Conclusion

With these investigations one could clearly show that flavonoids have a positive effect on the half-life of the *Hb*Hnl from *Hevea brasiliensis* and their stability. The stabilization seems to be a combination of radical scavenging, chelating of transition metals and some kind of steric effects. The contributions of the different effects to the half-life increase could not be determined. But it is clear that flavonoids are very well suited to improve the half-life of the *Hb*Hnl enzyme. The big advantage seems to be that only very small amounts are necessary to get a pronounced effect on the enzyme stability.

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Utjecaj flavonoida na stabilnost (S)-hidroksinitril-liaze iz *Hevea brasiliensis*

Sažetak

(S)-hidroksinitril-liaza iz listova kaučukovca *Hevea brasiliensis* (*Hb*Hnl) (EC 4.1.2.39) katalizira industrijski interesantnu sintezu (S)-cijanohidrina iz aldehida ili ketona i HCN. Ukupno iskorištenje na (S)-cijanohidrinu smanjuje se zbog nestereoselektivne povratne kemijske reakcije katalizirane bazama. Da bi se smanjila povratna kemijska reakcija, potrebno je raditi pri nižim pH-vrijednostima (ispod pH=5), ali je tada enzim nedovoljno stabilan. Da bi se enzim stabilizirao pod tim uvjetima, potrebno je dodati određene stabilizatore. Pokusi na stabilizaciji započeti su s ekstraktom iz listova *Hevea brasiliensis*. Listovi su ekstrahirani s puferom kalijeva citrata/fosfata, a dobiveni je ekstrakt kromatografski razdvojen u 5 frakcija. Ispitan je utjecaj ukupnog ekstrakta i dobivenih frakcija na poluživot enzima. Ekstrakt je produžio poluživot do 5 puta, a jedna od kromatografskih frakcija povisila je stabilnost za 50 %. Analizom te frakcije uočeno je da sadržava flavonoide. U nastavku radova ispitan je utjecaj čistih flavonoida na stabilnost enzima, te je nađeno da su male koncentracije rutina (5–20 ng mL^{-1}) i hiperosida (1,5–6 ng mL^{-1}) dovoljne kako bi povećale stabilnost enzima za 50 %. Nadalje, neki sintetski flavonoidi (Venoruton® i monohidroksietilrutosid) pokazali su također stabilizacijski učinak. Produženje poluživota bilo je čak veće od pet puta pri optimalnim uvjetima. Povećana stabilnost pripisuje se s jedne strane kelatnom učinku, a s druge utjecaju strukture, što se do sada ne može razlikovati.