Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 52–53 (2008) 128–132
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Secondary structure conformation of hydroperoxide lyase from green bell pepper, cloned in *Yarrowia lipolytica*, and its activity in selected media

Mirna P. Santiago-Gómez [a], Selim Kermasha [b], Jean-Marc Nicaud [c], Jean-Marc Belin [a], Florence Husson [a,*]

[a] *Laboratoire de Génie des Procédés Microbiologiques et Alimentaires (GPMA), Université de Bourgogne, Campus Universitaire Montmuzard, 1 Esplanade Erasme, 21000 Dijon, France*
[b] *Department of Food Science and Agricultural Chemistry, McGill University, 21,111 Lakeshore, Ste-Anne de Bellevue, Quebec, Canada H9X 3V9*
[c] *Laboratoire de Microbiologie et Génétique Moléculaire, INRA-CNRS-INAPG UMR 2585, BP 01, 78 850 Thiverval-Grignon, France*

Available online 19 November 2007

Abstract

Circular dichroism (CD) spectroscopy of secondary structure conformation of the purified green bell pepper hydroperoxide lyase (HPL), cloned in the yeast *Yarrowia lipolytica*, was investigated. The CD spectra of HPL in *iso*-octane, obtained at 60 °C, in the presence of the reducing agent dithiothreitol showed dramatic increase in α-helix content. The enzyme conformation remained unchanged over a range of pH values of 5.0–7.0. Using 13-hydroperoxide of linoleic acid (13-HPOD) as substrate, the biocatalysis of HPL in organic solvent media, including chloroform, dichloromethane, hexane, *iso*-octane, octane and toluene, was investigated. The results indicated an increase in HPL activity in the biphasic hexane medium.

Keywords: Hydroperoxide lyase; Secondary structure; Dichroism circular spectroscopy; Biocatalysis

1. Introduction

Hydroperoxide lyase (HPL, E.C. 4.1.2.), an enzyme widely found in plants and microorganisms, is involved in the biosynthesis of volatile aldehydes and alcohols. It catalyzes the breakdown of fatty acid hydroperoxides into oxoacids and aldehydes (C6 and C9), which are recognized as the fresh green odor of fruits and vegetables. Plant HPL is the major enzyme in the biosynthesis of natural volatile aldehydes; however, the recovery of the enzyme for its use in industrial applications is difficult [1]. The recombinant expression of the biocatalyst is the preferred way to obtain high quantities of stable and efficient enzyme [2].

The biocatalysis of enzymes in organic solvent media (OSM) is a useful approach when one or more components of the enzymatic reactions are poorly water-soluble [3]. Recently, the HPL activity was investigated in the presence of OSM, including hexane, methyl *tert*-butyl ether and acetonitrile, showing HPL activity in hexane and methyl *tert*-butyl ether, but not in acetonitrile [4]. The biocatalysis of purified soybean lipoxygenase (LOX), using linoleic acid as a model substrate, was also investigated in selected OSM, including chloroform, dichloromethane, hexane, *iso*-octane, octane and toluene [5]; the results indicated that there was an increase of 2.6-fold in LOX activity in the monophasic *iso*-octane medium compared to that obtained in the aqueous one. In addition, the experimental data obtained in these studies showed that the presence of a small volume of an organic solvent was necessary to enhance the enzymatic activity [5,6]; the same authors reported that several approaches have been used to investigate the enzymatic behavior in water-restricted environment, including the use of water miscible organic solvent systems [7,8], biphasic aqueous-organic solvent systems [9,10], reversed micelle systems [11] and monophasic organic solvent systems [12].

Circular dichroism (CD) spectra of HPL from alfalfa, cloned in the bacteria *Escherichia coli*, showed 75% of α-helix and there was no obvious structural differences in the presence or in the absence of 0.2% Triton X-100 [13]. In addition, CD studies at different temperature, pH and reaction media can be used for

* Corresponding author. Tel.: +33 03 80 39 66 80; fax: +33 03 80 39 66 41.
E-mail address: florence.husson@u-bourgogne.fr (F. Husson).

1381-1177/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2007.11.014

providing information on the stability of the proteins [14]. CD is also an effective tool in monitoring the changes in the secondary and tertiary structures of protein binding [15,16].

The objective of this research was to study the conformation of the secondary structure of HPL from green bell pepper cloned in the yeast *Yarrowia lipolytica,* by CD spectroscopy, at different conditions of enzymatic reaction. In addition, the HPL activity and its secondary conformation in OSM at different temperatures and pH values were investigated.

2. Materials and methods

2.1. Culture of yeast

The strain JMY 861 of *Y. lipolytica* expressing a 6-His-tagged (N-terminal) green bell pepper HPO lyase was previously constructed by Bourel et al. [17]. It was grown on YTGA (5 g/L yeast extract, 10 g/L tryptone, 10 g/L glucose, 15 g/L agar) medium at 27 °C for 48 h. The biomass was harvested and inoculated in 100 mL YTG (5 g/L yeast extract, 10 g/L tryptone, 10 g/L glucose) liquid medium for 24 h. The volume of the suspension was determined in order to have an initial OD_{600} of 0.2. After 24 h of pre-culture, the biomass was inoculated with an initial OD_{600} of 4 (10^7 cells/mL) in 100 mL YTO medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L olive oil, 5.3 g/L NH_4Cl). The cells were grown in 500 mL baffled Erlenmeyer flasks, agitated on a rotary shaker at 140 rpm and 27 °C. The biomass was then harvested for its use as source of HPL.

2.2. HPL extraction and purification

All steps of extraction and recovery were carried out at 4 °C, unless otherwise indicated. The biomass of the yeast was washed three times with Tris–HCl buffer (100 mM, pH 8.0). The washed cells were suspended in the same buffer, but also containing 2% Triton X-100R (for protein extraction) (Sigma Chemical Co.; St-Louis, MO, USA), 0.3 M NaCl, 2 mM imidazole and EDTA-free protease Inhibitor Cocktail Tablets (Roche Diagnostics, Penzberg, Germany), using one tablet per 50 mL of the Tris–HCl buffer or 1 g per 6 mL buffer. The cell suspension was homogenized using a one-shot cell disrupter (Z plus series "One shot") at 1.6 kbar. The homogenized disrupted cell suspension was centrifuged (10,000 × *g*, 20 min) three times and the supernatant was considered to be the crude enzymatic extract.

Three millilitres of the resin Ni-NTA Agarose (QUIAGEN, Courtaboeuf, France) was preliminary equilibrated on column ($d = 1$ cm, $h = 1$ cm) with 15 mL of buffer A (Tris–HCl, 100 mM, pH 8.0), containing 0.2% Triton X-100R, 0.3 M NaCl and 2 mM imidazole, and at a flow rate of 0.5 mL/min. Thirty millilitres of crude enzymatic extract were loaded onto the equilibrated resin at a flow rate of 0.3 mL/min. The resin was then washed with 15 mL of buffer A and 15 mL of buffer B (sodium phosphate, 50 mM, pH 6.0), containing 0.2% Triton X-100R, 0.3 M NaCl and, 15 mM imidazole, and at a flow rate of 0.5 mL/min. The His-tagged protein was eluted with 30 mL buffer C (sodium phosphate, 50 mM, pH 5.0, containing 250 mM imidazole), dialyzed for the removal of imidazole and Triton X-100R and finally concentrated by using Microcon Centrifugal Filter devise (Millipore, France). The protein concentration was determinate using a calculated A_{280} coefficient. The numbers of Trp, Tyr and Phe residues were deduced from the HPL gene sequence, published by Matsui et al. [18].

2.3. HPL enzymatic assay

HPL activity was determined using potassium phosphate buffer solution (100 mM, pH 5.5), containing 25 mM of 13-hydroperoxide of linoleic acid (13-HPOD) as substrate, prepared in absolute ethanol. The enzymatic reaction was initiated by the addition of 4 μL of the purified enzymatic protein (0.7 mg/mL). The biphasic organic solvent system consisted of (0–5%, v/v) of one of the selected organic solvents, including *iso*-octane, *n*-hexane, dichloromethane, octane, toluene and chloroform, in potassium phosphate buffer (100 mM, pH 5.5). The decrease in A_{234}, due to the cleavage of the substrate, was followed spectrophotometrically for 1 min. The activity was calculated from the initial slope of the resulting absorbance curve. One unit of HPL activity was defined as the amount of enzyme that converted 1 μmol of HOPD substrate within 1 min.

2.4. Circular dichroism (CD)

CD spectra were recorded on a Jobin–Yvon spectropolarimeter (Longjumeau, France), using 5 μM of the purified HPL. The CD spectra were analyzed with the DICHROPROT V2.5 application package [19] to estimate the secondary structure composition. Protein samples were suspended in potassium phosphate buffer (100 mM, pH 5.5) and scanned at different temperatures (5, 20, 40 and 60 °C) or at different pH (5.0, 5.5, 6.0 and 7.0) and scanned at 20 °C. The reaction medium contained 3% of one of the selected organic solvents as well as dithiothreitol (DTT), as a reducing agent, and potassium ferricyanide, as an oxidizing agent. For the measurement, quartz Suprasil cuvettes, with a path length of 1 cm, were used. The spectra were recorded in the wavelength range of 180–260 nm, in $\Delta\varepsilon\,M^{-1}\,cm^{-1}$ for residue in function of path length λ nm. The path intensity, at 222 nm, was set in a manner to estimate the helices percentage in the polypeptide: $P\alpha = -[\ddot{A}\mathring{a}_{222} \times 10]$ ($P\alpha$: helices α percentage; $\ddot{A}\mathring{a}_{222}$: dichroic increment for residue at 222 nm) [20].

3. Results and discussion

3.1. HPL activity in organic solvent media

HPL activity was investigated in various biphasic systems, containing potassium phosphate buffer solution (100 mM, pH 5.5) and 1% of one of the selected organic solvents. For the control assays, only buffer solution was used. The results in Table 1 showed an increase in HPL activity of 1.50, 1.50 and 1.28 folds, when *iso*-octane, *n*-hexane and dichloromethane were used, respectively, in the reaction medium as compared to that obtained in the aqueous one. The maximum enzymatic activity was found in the *iso*-octane and hexane biphasic sys-

Table 1
HPL in biphasic organic solvent systems

Biphasic system	HPL activity purified enzyme (U/mL)
Control	0.85 (0.03)
Iso-Octane	1.27 (±0.15)
n-Hexane	1.26 (±0.38)
Dichloromethane	1.09 (±0.28)
Octane	0.77 (±0.19)
Toluene	0.29 (±0.06)
Chloroform	-

All the experiments were carried out three times independently, the data represented the mean and the standard deviation.

tems. The experimental results indicated a negative effect of octane and toluene on the enzymatic activity. Similar results were reported [5] for the biocatalysis of purified soybean lipoxygenase (LOX); these authors indicated an increase of 4.7-fold in LOX activity when the enzymatic reaction was performed in a medium containing 4 % of *iso*-octane, and an increase of 1.5-fold in media containing one of the following organic solvents, 1% dichloromethane 1% hexane and 3.5% octane. Kermasha et al. [5] also reported a dramatic decrease in LOX activity when the reaction was performed in a medium containing 1% of chloroform or toluene; these results suggested that the presence of small amount of non-polar organic solvents in aqueous medium could result by an increase in the solubility of the hydrophobic substrate, hydroperoxides, so that the interaction between the enzyme and the substrate is enhanced.

HPL activity was investigated, using a wide range of concentrations (0–5%) of *iso*-octane, *n*-hexane and dichloromethane. The results in Fig. 1 show that the use of 3% *n*-hexane or *iso*-octane increased the HPL activity from 0.85 to 2.20 U/mL and 0.85 to 1.70 U/mL, respectively, as compared to that obtained in aqueous medium. However, there was no significant change with the use of octane. Several authors [12,21,22] indicated that the polar organic solvents strip the water from the enzymes, leading to the unfolding of the protein molecule, with the exposure of the inner hydrophobic residues; this will lead to the denaturation at a much faster rate than that in a pure aqueous system.

3.2. Determination of HPL secondary conformation

In order to correlate the HPL activity with its secondary conformation, CD spectra of purified HPL were recorded in several conditions of temperature, pH of aqueous buffer, in reducing and oxidizing conditions and with organic solvents. These conditions were proved to be important for hexanal production and HPL activity [23].

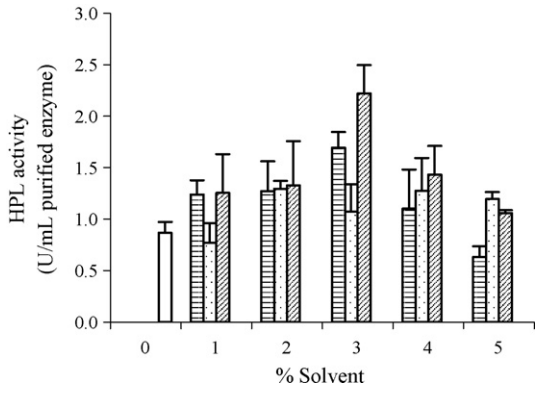

Fig. 1. HPL activity in different reaction media containing potassium phosphate (100 M, pH 5.5) and organic solvents: control (□), *iso*-octane (≡), octane (▒) and *n*-hexane ///. All the experiments were carried out three times independently, the data represented the mean and the standard deviation.

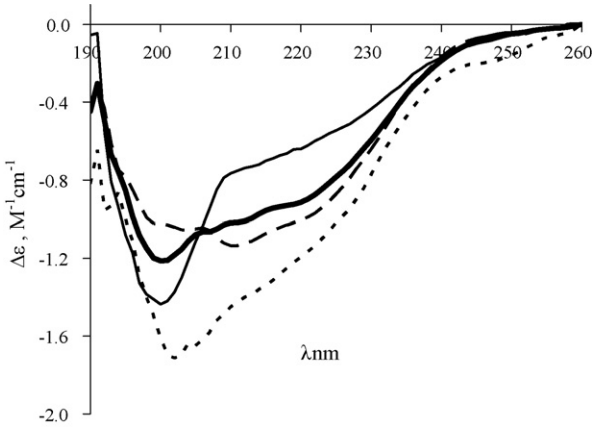

Fig. 2. CD spectra of bell pepper HPL obtained at different temperatures: 5 °C (––), 20 °C (▬), 40 °C (. . .) and 60 °C (–) in potassium phosphate buffer 100 mM (pH 5.5).

3.2.1. Effect of temperature on the HPL secondary conformation

CD spectra (Fig. 2) of the purified HPL were recorded in potassium phosphate buffer solution (100 mM, pH 5.5), using a range of temperatures from 5 to 60 °C. The results in Table 2 indicate an increase in α-helix, from 6% to 9%, when the HPL was heated at 60 °C, indicating hence that subjecting the enzyme to heat treatment did result by the unfolding of its protein con-

Table 2
Secondary structure distribution of bell pepper HPL in different reaction media

Structure	Temperature (°C)				pH			Molecules		Solvent (3%)	
	5	20[a]	40	60	5	6	7	DTT	$K_3Fe(CN)_6$	*n*-Hexane	*Iso*-octane
α-Helix	6	7	7	9	6	7	6	10	-	7	8
β-Sheet	28	26	28	19	30	28	32	26	-	29	28
Turn	7	7	6	4	6	6	6	6	-	6	6
Random coil	58	60	59	68	58	58	56	58	-	58	58

[a] Control.

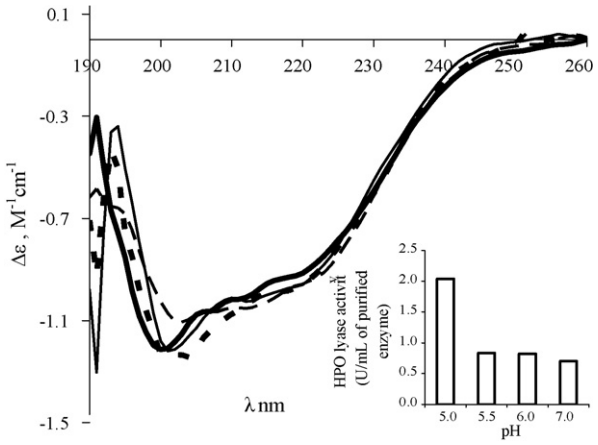

Fig. 3. CD spectra of bell pepper HPL obtained at different pH: 5 (––), 5.5 (▬), 6 (. . .) and 7 (–); the CD spectra and the HPL activity was measured in potassium phosphate buffer 100 mM at 20 °C.

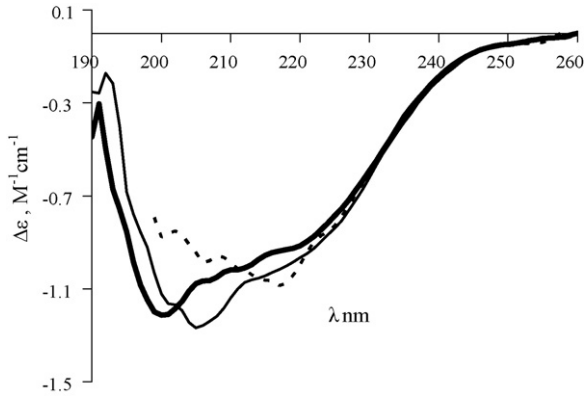

Fig. 4. CD spectra of bell pepper HPL measured in potassium phosphate buffer 100 mM (pH 5.5) containing dithiothreitol 0.5 mM (–) and potassium ferricyanide 1 mM (. . .) at 20 °C. The control (▬) was measured in a potassium phosphate buffer 100 mM (pH 5.5).

formation; this slight increase in α-helix is in agreement with the results obtained for the secondary structure of bovine casein [24], where an apparent increase in α-helix, from 15% to 22%, when the temperature was increased from 2 to 70 °C. On the other hand, the results in Table 2 indicate a decrease in β-sheet and an increase in random coil at 60 °C.

3.2.2. Effect of pH on the HPO lyase secondary structure

When potassium phosphate buffer solution (20 °C) was adjusted at different pH values, 5.0, 5.5, 6.0 and 7.0, a limited decrease (1%) in α-helix was obtained at pH 5.0 and 7.0 (Fig. 3). On the other hand, an increase from 30% at pH 5 to 32% at pH 7.0, in β-sheet was obtained (Table 2). However, there was more than 50% decrease of HPL activity when the pH was higher than 5.0 (Fig. 3). Similar results have been reported [25] for a purified lipase B from *Candida antarctica*, using a wide range of buffer solutions of pH values ranging from 4.2 to 9.0. A decrease in the α-helix was obtained at the extremes of the pH range, with 32% at pH 4.2, 37% at pH 6.0 and 30% at pH 9.0; however, the remaining enzymatic protein structure did not appear to be changed significantly over the investigated pH values. Moreover, these authors [25] concluded that a significant decrease in α-helix content at the extreme pH, 4.3 and 9.0, explain the decrease in enzymatic activity. The relationship between the decrease in HPL activity and the increase in pH value could not be explained by a dramatic change in the secondary structure of the enzyme; the decrease in HPL activity at pH higher than 5.0 could be explained by the direct effect of the pH on the ionization of key residues, in particular the cysteine, at the active site of the enzyme.

3.2.3. Effects of dithiothreitol and potassium ferricyanide on HPL secondary structure

Fig. 4 shows the CD spectra of the purified HPL, in potassium phosphate buffer solution (100 mM, pH 5.5), containing 0.5 mM dithiothreitol as the reducing agent or 1 mM potassium ferricyanide as the oxidizing one. The results indicated a significant increase, from 7% to 10%, in α-helix of the enzyme structure in reducing conditions. In a previous work, carried out by our group [23], the experimental findings showed an increase in HPL activity (1.9–2.16 U/mL), in the presence of 0.5 mM DTT; however, the results also showed a decrease in enzyme activity in the presence 1 mM potassium ferricyanide. In addition to its role as the reduced redox potential of the culture medium (E_h = +178 mV), DTT effect was indebted to its direct molecular effect on HPL through its role as antioxidant, protecting hence the thiol function at the active site of the enzyme [25]. In this study, the results confirmed the role of DTT as a reducing agent and the percentage of HPL α-helix in its presence was enhanced from 7% to 10%. It is known that DTT breaks the disulfide bridges and maintains the cys side chains in their reduced state [26], unfolding hence the protein structure.

3.2.4. Effects of organic solvents on HPL secondary structure

Fig. 5 shows the CD spectra, obtained in a potassium phosphate buffer (100 mM, pH 5.5), containing 3% of *n*-hexane and *iso*-octane. The results in Table 2 indicate that a slight increase of 1% in α-helix and 2% in β-sheet was obtained with the presence

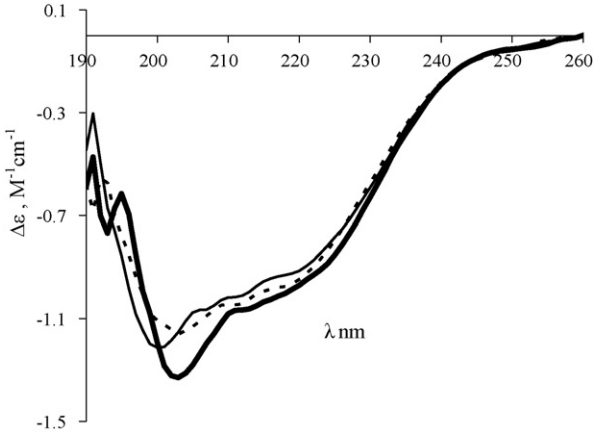

Fig. 5. CD spectra of bell pepper HPL measured in potassium phosphate buffer 100 mM (pH 5.5) containing *iso*-octane (–) and *n*-hexane (. . .) 3% at 20 °C. The control (▬) was measured in a potassium phosphate buffer 100 mM (pH 5.5).

of *iso*-octane as compared to that in the control buffer solution. With the presence of *n*-hexane, there was an increase of 3% in β-sheet. In addition, a decrease in the random coil was founded in the presence of *n*-hexane and *iso*-octane. These experimental findings are in agreement with the explanations given by McCabe et al. [25] who indicated that the secondary structures of enzymes in organic solvent, determined by CD spectroscopy, showed great differences as compared to those obtained in aqueous medium. While one might expect the proteins to unfold in most organic solvents because of their inner hydrophobic nature, their stability may be increased in the hydrophobic organic solvents because of the reduced mobility of the chains of proteins.

4. Conclusion

CD spectra of purified green bell pepper HPL, cloned in *Y. lipolytica*, were determined in different conditions of temperature, pH, reducing and oxidizing molecules and solvents. The secondary structure contents were quantitatively analyzed and it was founded that HPL structure remained relatively stable in all investigated conditions. Indeed, a significant increase in enzyme activity was obtained in two particular conditions, 3% *n*-hexane and 1% DTT, where a significant increase in α-helix was monitored.

Acknowledgements

Mirna P. Santiago-Gómez was awarded a fellowship, for her graduate studies, by the Mexican Council for Science and Technology (CONACYT).

References

[1] J. Delcarte, M.-L. Fauconnier, P. Jacques, K. Matsui, P. Thonart, M. Marlier, J. Chromatogr. B 786 (2003) 229.
[2] K. Matsui, C. Ujita, S-h. Fujimoto, J. Wilkinson, B. Hiatt, V. Knauf, T. Kajiwara, I. Feussner, FEBS Lett. 481 (2000) 183.
[3] G.J. Piazza, Biotechnol. Lett. 14 (1992) 1153.
[4] J.-L. Dominick, L.L. Raymond, J. Mol Catal. B: Enzym. 44 (2006) 32.
[5] S. Kermasha, N. Dioum, B. Bisakowski, J. Mol. Catal. B: Enzym. 11 (2001) 909.
[6] S. Kermasha, N. Dioum, B. Bisakowski, M. Vega, J. Mol. Catal. B: Enzym. 19–20 (2002) 305.
[7] M.D. Lilly, J.M. Woodley, in: C. Laane, J. Tramper, M.D. Lilly (Eds.), Biocatalysis in Organic Media, Elsevier, Amsterdam, 1987, p. 179.
[8] G. Carrea, in: C. Laane, J. Tramper, M.D. Lilly (Eds.), Biocatalysis in Organic Media, Elsevier, Amterdam, 1987, p. 157.
[9] G. Carrea, Trend Biotechnol. 2 (1986) 102.
[10] M.D. Lilly, J. Chem. Technol. Biotechnol. 32 (1982) 162.
[11] K. Martinek, A.N. Semenov, I.V. Berezin, Biochim. Biophys. Acta 658 (1986) 76.
[12] A.M. Klibanov, Chemtech 16 (1986) 354.
[13] M.A. Noordermeer, G.A. Veldink, J.F.G. Vliegenthart, FEBS Lett. 489 (2001) 229.
[14] S.M. Kelly, T.J. Jess, N.C. Price, Biochim. Biophys. Acta 1751 (2005) 119.
[15] N.J. Greenfiel, Anal. Biochem. 235 (1996) 1.
[16] G.D. Fasman, Plenum. New York, 1996, p. 69.
[17] G. Bourel, J.M. Nicaud, B. Nthangeni, P. Santiago-Gomez, J.M. Belin, F. Husson, Enzym. Microb. Technol. 35 (2004) 293–299.
[18] K. Matsui, M. Shibutani, T. Hase, T. Kajiwara, FEBS Lett. 394 (1996) 21.
[19] G. Deléage, C. Geourjon, Appl. Biosci. 9 (1993) 197.
[20] L. Zhong, W.C. Johnson, Biophysics 89 (1992) 4462.
[21] V.M.G. Lima, N. Krieger, D.A. Mitchell, J.D. Fontana, Biochem. Eng. J. 18 (2004) 65.
[22] A.M. Azevedo, D.M.F. Prazeres, J.M.S. Cabral, L.P. Fonseca, J. Mol. Catal. B: Enzym. (2001) 147.
[23] F. Husson, V.P. Tu, M. Santiago-Gomez, R. Cachon, G. Feron, J.-M. Nicaud, S. Kermasha, J.-M. Belin, J. Mol. Catal. B: Enzym. 39 (2006) 179.
[24] H.M. Farell Jr., E.D. Wickham, J.J. Unruh, P.X. Qi, P.D. Hoagland, Food Hydrocolloid. 15 (2001) 341.
[25] R.W. McCabe, A. Rodger, A. Taylor, Enzym. Microb. Technol. 36 (2005) 70.
[26] W.J. Wolf, J. Agr. Food Chem. 41 (1993) 168.