FEBS Letters 441 (1998) 348–352 FEBS 21329

Proposition for the biochemical mechanism occurring in the sucrose isomerase active site

Thierry Veronese, Patrice Perlot*

Eridania-Béghin-Say, Institut National des Sciences Appliquées, Hall Gilbert Durand, Avenue de Rangueil, 31077 Toulouse Cedex, France

Received 21 October 1998

Abstract Sucrose conversion by sucrose isomerase from *Protaminobacter rubrum*, *Serratia plymuthica* and *Erwinia rhapontici* was investigated in the presence of different monosaccharides in the reaction mixture. These conditions led to inhibitory effects and to glucosyl transfer of the glucose moiety of sucrose to the exogen monosaccharide. Comparison of the structure of the different inhibitors and acceptors has allowed us to suppose the binding of the sucrose molecule into the active site, and thereafter, to propose the possible biochemical reactions leading to the formation of different products from sucrose. This study also underlines the close homologies between these enzymes.

Key words: Isomaltulose; Trehalulose; Sucrose isomerase; Isomaltulose synthase

1. Introduction

In the past decades, the production of isomaltulose (6-*O*-α-D-glucopyranosyl-D-fructofuranose) and trehalulose (1-*O*-α-D-glucopyranosyl-D-fructose) has aroused great interest since these structural isomers of sucrose have interesting potentialities as acariogenic sweeteners [1,2]. Various isomaltulose-producing microorganisms have been used industrially for the production of these sugars because of their ability to produce a particular α-glucosyltransferase (a sucrose isomerase) which catalyses the isomerisation of sucrose into both isomaltulose and trehalulose. The purification of such an enzyme was performed from the microorganisms *Protaminobacter rubrum* [3], *Erwinia rhapontici* [4], *Serratia plymuthica* [5], *Pseudomonas mesoacidophila* [6] and *Klebsiella planticola* [7]. Very few studies have investigated the mechanism of such enzymes except Cheetham in 1983, who proposed an explanation for the formation of the two products by the sucrose isomerase from *E. rhapontici* [4] by suggesting the sucrose conversion by a true intramolecular mechanism in a closed shell process.

In order to contribute to the understanding of this mechanism, we focused our attention on *Serratia plymuthica* sucrose isomerase and we found the production of several products in addition to isomaltulose and trehalulose formation (i.e. isomaltose, isomelezitose, glucose and fructose). Therefore, we proceeded to several experiments which showed that product distribution was largely depending on temperature and on fructose and glucose addition to the reaction mixture. Finally, we proposed an explanation to this result by a mechanism combining intra- and intermolecular reactions [5]. In our laboratory, we have also performed the same investigations on *Erwinia rhapontici*, *Klebsiella terrigena* and *Protaminobacter rubrum* sucrose isomerase, and these studies led to the same type of results, demonstrating that all these sucrose isomerases act with a common mechanism.

In this paper, our objective is to complete this mechanism by giving an approach of the reaction occurring at the active site level. As a result, we propose a biochemical scheme of the possible binding of the sucrose molecule and of the different inter- and intramolecular reactions.

2. Materials and methods

2.1. Sucrose isomerases

All studies were performed with pure sucrose isomerases from *Serratia plymuthica*, *Protaminobacter rubrum*, and *Erwinia rhapontici*. The production and purification of these proteins have been performed according to the method described in our previous publication [5].

2.2. Sugars

All sugars were supplied by Sigma (St. Louis, MO, USA).

2.3. Standard assay of sucrose isomerase activity

Enzyme activity was measured by incubating 100 μl of purified enzyme solution with 900 μl of sucrose solution at a final concentration of 292 mM (100 g/l), in 50 mM phosphate buffer (pH 6.2). The reaction temperature was 30°C for the sucrose isomerase from *P. rubrum* and *S. plymuthica*, and 35°C for the sucrose isomerase from *E. rhapontici*. Several samplings of the reaction mixture were done, and the activity was calculated by plotting product formation vs. reaction time. The international unit activity (UI) is defined as the amount of enzyme that can convert one μmol of sucrose per minute at the initial stage under the standard assay conditions.

2.4. Sucrose conversion in the presence of different monosaccharides

We performed several experiments by incubating each purified enzyme in the standard assay conditions but with adding alternately 277 mM of different monosaccharides. This procedure was done in order to investigate the ability of sucrose isomerases to transfer glucose from sucrose to an exogen acceptor, and the kinetics of transfer was calculated by measuring the production of the glucosyl-acceptor product vs. time. We defined one unit (U) activity as the amount of enzyme that can produce one μmol of glucosyl acceptor per minute in the standard assay conditions. Thus, the relative transfer activity was estimated by the relation:

$$\text{Relative transfer} = \frac{\text{transfer activity} \times 100}{\text{total enzyme activity}}$$

In parallel, we also measured the inhibitory effect of the monosaccharides added to the reaction mixture. The relative inhibition was calculated as follows:

$$\text{Relative inhibition} = 100 - \frac{\text{total enzyme activity with inhibitor} \times 100}{\text{total enzyme activity without inhibitor}}$$

2.5. Sugar analysis

Samples were delivered by an ICS 1708 autosampler with 10 μl fixed loop to a Dionex DX40 chromatography system (Dionex). Separation was carried out in a 4×250-mm Dionex Carbo-pack PA100 column preceded by a 3×25-mm Dionex Carbo-pack PA100 Guard

*Corresponding author. Fax: (33) (5) 61 55 94 70.

PII: S0014-5793(98)01582-8

column. Mobile phase was propelled by a Dionex GP40 quaternary pump at a 1-ml/min flow rate. It was composed of a two eluent mix containing nanopure water and 0.15 M NaOH. Both eluents were degassed by helium sparging in a Dionex degassing module. Detection was done by a Dionex ED40 module with a gold working electrode and a pH Ag/AgCl reference. Sensitivity was set at 10 μC. Potentials for amperometric detection were applied as follows: delay time $t_1 = 200$ ms, potential $E_1 = 50$ mV; electrode oxidation by the sample, $t_2 = 200$ ms, $E_2 = 50$ mV; electrode cleaning $t_3 = 200$ ms, $E_3 = 750$ mV; electrode reduction $t_4 = 400$ ms, $E_4 = -150$ mV.

3. Results and discussion

Table 1 shows the relative inhibitor effect and the relative transfer activity obtained for each sucrose isomerase incubated with sucrose and with different monosaccharides. We based our reasoning on these results to suggest a completion of the mechanism we have designed in our previous studies [5]. Our purpose is to propose a biochemical approach at the sucrose-isomerase active site level of the different reaction we have suggested for this mechanism (for more precision and justification, refer to our previous publication [5]). As these experiments have lead to very similar results for the three sucrose isomerases we have studied, we mention a single reasoning which is then valid for each enzyme. The manner in which sucrose would bind to the active site has been deduced from the following observations.

3.1. Preferential structure of the inhibitor and deduction of binding of the glucose moiety of sucrose

In our previous studies [5], we have demonstrated that glucose is a competitive inhibitor of sucrose. Thus, we deduced the binding of the glucose moiety of sucrose in the active site by assuming that free glucose binds to the enzyme in the same site and in the same way. Then, by comparing with glucose, the potential inhibition and the structure of different monosaccharides, we estimated the relative importance of the presence and the orientation of each hydroxyl group of the glucose molecule to allow this binding. For better comprehension, refer to Fig. 1 which shows the chemical structure of the molecules tested. The results shown in Table 1 demonstrate that 2-OH and 3-OH are necessary for glucose binding since 2- and 3-deoxy-glucose are less inhibitor. Moreover, the test with mannose tends to prove that 2-OH must be in equatorial position, and this is confirmed by comparing the results with xylose and lyxose. In the same way, 3-OH in axial position seems to affect the binding since poor inhibition is obtained with allose or gulose. Concerning 4-OH, this hydroxyl seems to be useful in equatorial position since galactose or fucose are both less inhibitors than glucose. By comparing the results with these two latter sugars, and referring to their structure, we deduced also that 6-OH is certainly not involved in the binding. This is supported by the fact that 6-deoxy- or 6-chloro-glucose are very good inhibitors. Finally, the role of 1-OH can be deduced from the fact that this hydroxyl is present in the whole monosaccharide tested except in the fructopyranose and tagatose molecules which do not lead to inhibition in most cases (however, the representation of tagatose has been orientated in order to exhibit the 6-OH of this molecule at the same position as the 1-OH of glucose). We concluded thereby that this hydroxyl, which is involved in the glycosidic bond with the fructose moiety in the sucrose molecule, is also important for the binding of glucose.

3.2. Preferential structure of the acceptor molecule and deduction of the binding of the fructose moiety of sucrose

The properties of sucrose isomerases to transfer the glucose moiety from sucrose to an exogen acceptor was used to deduce the manner in which sucrose binds to the active site via its fructose moiety. The molecules tested are the same we have used to investigate the potential inhibitors but Fig. 1 shows the structure of these molecules in an orientation compatible with their possible insertion in the active site. Table 1 shows that the acceptor must exhibit two hydroxyl groups equivalent to the 2-OH and 3-OH of glucose since we obtained less transfer by using 2-deoxy-glucose, and very poor transfer with 3-deoxy-glucose. Axial or equatorial position for 2-OH do not seem to be important since mannose is very well transferred, whereas equatorial position for 3-OH is significant since allose is not transferred. As galactose is much less transferred than glucose, we deduced that 4-OH must preferably be in equatorial position. The key role of 6-OH on transfer reaction was demonstrated. Indeed, isomaltose production by

Table 1
Effect of the addition of different monosaccharides to the reaction mixture on sucrose-isomerase activity

Sugars tested	Relative inhibition (%)			Relative transfer (%)		
	A	B	C	A	B	C
α-D-Glucose	50	77	40	3.5	3.0	5.6
2-Deoxy-α-D-glucose	20	0	10	2.7	4.0	4.3
3-Deoxy-α-D-glucose	0	25	10	0.6	0.6	2.0
6-Deoxy-α-D-glucose	70	78	70	0.0	0.0	0.0
6-Chloro-α-D-glucose	65	52	50	0.0	0.0	0.0
1-*O*-Methyl-α-D-glucose	60	42	55	0.0	0.0	0.0
β-D-Fructofuranose	0	0	0	n.d.	n.d.	n.d.
β-D-Fructopyranose	0	0	0	n.d.	n.d.	n.d.
α-D-Galactose	20	0	15	0.8	1.0	1.0
α-D-Fucose	20	15	15	0.0	0.0	0.0
α-D-Mannose	9	0	7	3.3	5.0	4.9
α-D-Xylose	40	23	30	0.0	0.0	0.0
α-D-Lyxose	0	0	0	0.0	0.0	0.0
β-D-Allose	25	23	8	0.0	0.0	0.0
α-D-Gulose	5	20	30	2.1	2.8	3.6
β-D-Tagatose	0	0	0	3.5	5.6	4.5

n.d.: Not detectable. A: Sucrose isomerase from *Erwinia rhapontici*; B: sucrose isomerase from *Protaminobacter rubrum*; C: sucrose isomerase from *Serratia plymuthica*.

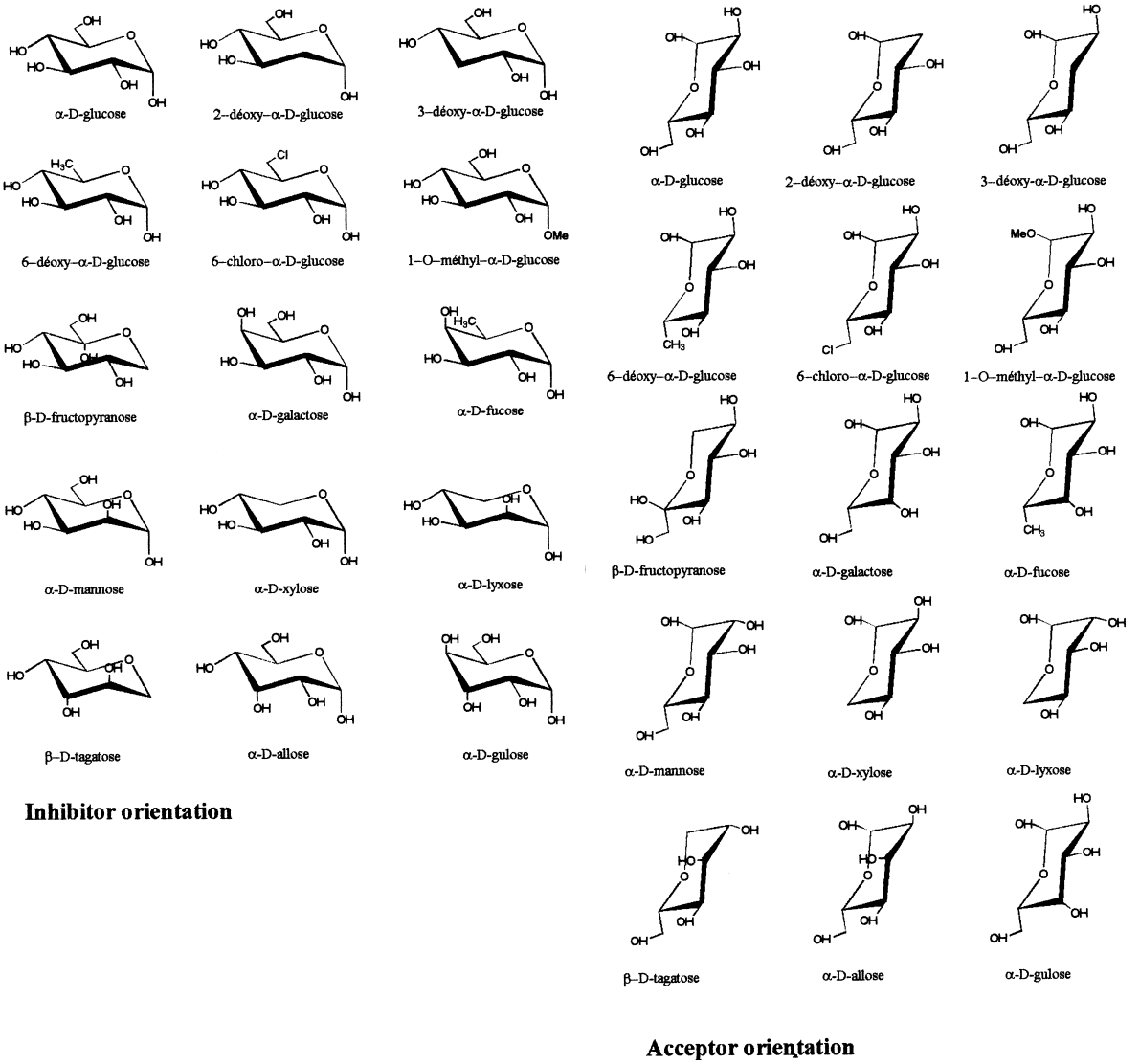

Fig. 1. Structure of the monosaccharides tested.

sucrose isomerase is the result of a transfer reaction from the glucose moiety of sucrose to the 6-OH of an exogen molecule of glucose. Our experiments show that transfer cannot be obtained if this hydroxyl is not present since 6-chloro- or 6-deoxy-glucose were unreactive. Furthermore, all the molecules which were transferred exhibit such a primary hydroxyl in the sixth position as shown in Fig. 1 (i.e. 2- and 3-deoxy-glucose, galactose, mannose, gulose and tagatose for which the 'sixth' hydroxyl is in fact the first one). This is confirmed by comparing the results with galactose and 6-deoxy-galactose (i.e. fucose) and by noticing that xylose and lyxose are effectively not transferred. Finally, we must mention that fructose addition to the reaction mixture leads to additional trehalulose production, and we can assume that this phenomenon is due to the transfer of glucose to exogen fructopyranose, which is the preponderant form of fructose in solution [8]. We can observe in Fig. 2 the close structural homologies of the glucose and fructopyranose molecules. However, fructofuranose can also be an acceptor and allows a slight increase in isomaltulose production. Such transfer of fructose to form isomaltulose can be clearly visible by using 1-fluoro-fructose as acceptor. Unsurprisingly, we obtained additional isomaltulose (certainly under its 1′-fluoro-isomaltulose form), but no additional trehalulose formation was detected.

These results have allowed us to design the sucrose-isomerase mechanism with a biochemical approach at the active site level (Fig. 2). The structure of this latter was predicted by the possible binding of sucrose we have demonstrated above. As the transfer reaction proceeds via a glucosyl-enzyme intermediate and generates transfer products without modifying the configuration of the anomeric carbon, the reactive group of this site was inspired from α-glycosidases since these enzymes act with such a mechanism [9]. In this scheme, molecules of sucrose, isomaltulose and trehalulose are shown according to their conformation in water [8,10]. Each reaction is numbered as follows: 1: binding of the inhibitor (glucose in this case, but it can be replaced by one of the inhibitors shown in Fig. 1); 2: binding of sucrose; 3: sucrose hydrolysis; 4: isomaltulose formation; 5: opening of the fructofuranose ring; 6: fructofuranose tautomerisation into fructopyranose; 7: trehalulose formation; 8: fructopyranose release (glucosyl-enzyme formation); 9: fructofuranose release (glucosyl-en-

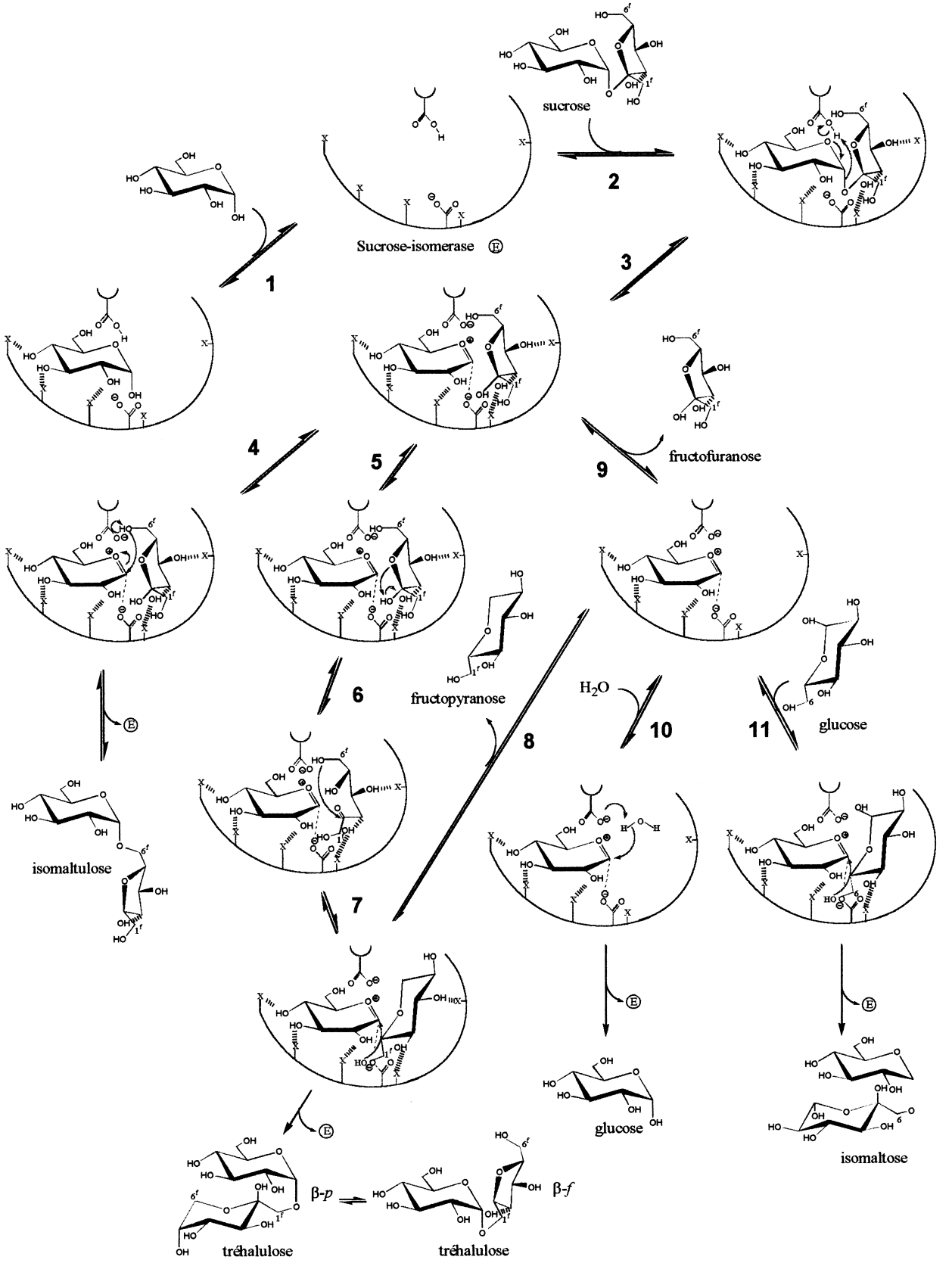

Fig. 2. Possible mechanism occurring in the sucrose-isomerase active site.

zyme formation); 10: glucose formation by transfer on a water molecule; 11: insertion of the acceptor in the active site (glucose in this case); transfer product formation (isomaltose in this case, but it can be replaced by one of the acceptors shown in Fig. 1).

References

[1] Minami, T., Fujiwara, T., Ooshima, T., Nakajima, Y. and Hamada, S. (1990) Oral Microbiol. Immunol. 5, 189–194.
[2] Ooshima, T., Izumitani, A., Minami, T., Fujiwara, T., Nakajima, Y. and Hamada, S. (1991) Caries Res. 25, 277–282.
[3] Nakajima, Y. (1988) Denpun Kagaku (J. Jpn. Soc. Starch Sci.) 35, 131–139 (in Japanese).
[4] Cheetam, P.S.J. (1983) Biochem. J. 220, 213–220.
[5] Véronèse, T. and Perlot, P. (1998) Enzyme Microbiol. Technol., in press.
[6] Nagai, Y., Sugitani, T. and Tsuyuki, K. (1994) Biosci. Biotech. Biochem. 58, 1789–1793.
[7] Ideno, A., Kishuiara, S., Fujii, S., Sugitani, T., Tsuyuki, K. and Ebashi, T. (1991) Abstracts of Papers, The Annual Meeting of the Japan Society for Bioscience, Biotechnology and Agrochemistry, Kyoto, April 1991, pp. 284.
[8] Lichtenthaler, F.W. and Rönninger, S. (1990) J. Chem. Soc. Perkin Trans. 2, 1489–1497.
[9] Ichikawa, Y., Look, G.C. and Wong, C. (1992) Anal. Biochem. 202, 215–238.
[10] Immel, S. and Lichthenthaler, F.W. (1995) Liebigs Ann., 1925–1937.