ELSEVIER

Carbohydrate Research 277 (1995) 161–169

CARBOHYDRATE RESEARCH

Partial purification and characterization of nitrophenyl maltosaccharide-hydrolyzing enzymes from *Lactobacillus* sp. no. 26X

Hans Bender

Institut für Organische Chemie und Biochemie der Universität Freiburg i.Br., Albertstrasse 21, D-79104 Freiburg i.Br., Germany

Received 27 February 1995; accepted 2 May 1995

Abstract

Two nitrophenyl maltosaccharide-hydrolyzing enzymes were partially purified from *Lactobacillus* sp. no. 26X. One of the enzymes, designated nitrophenyl α-maltosidase, being a homotetramer with M_r ca. 163,000, preferentially acted on *p*-nitrophenyl α-maltoside. The hydrolysis rates decreased with increasing length of the nitrophenyl maltosaccharides. Maltose, maltotriose, maltotetraose, and *p*-nitrophenyl α-D-glucopyranoside were scarcely attacked. The enzyme mainly hydrolyzed the bond between the nitrophenyl group and the sugar moiety, and *o*- and *p*-nitrophenylated substrates were hydrolyzed equally well. The second enzyme, designated nitrophenyl α-D-glucosidase, was presumably a homodimer with M_r ca. 51,000, and was specific for *p*-nitrophenyl α-D-glucopyranoside. The usefulness of the nitrophenyl α-maltosidase for coupled enzymic assays for the decycling maltodextrinase and alpha-amylases with nitrophenyl maltosaccharides as the substrates is discussed.

Keywords: *Lactobacillus* sp. 26X; Nitrophenyl maltosaccharide-hydrolyzing enzymes; Substrate specifity; Coupled enzymic assays for the decycling maltodextrinase and alpha-amylases

1. Introduction

Nitrophenyl maltosaccharides ($NP\alpha G_n$) such as *p*-nitrophenyl α-maltopentaoside, -maltohexaoside, and -maltoheptaoside ($pNP\alpha G_5$–$pNP\alpha G_7$) have been proposed [1,2] as substrates for coupled enzymic assays for alpha-amylases (EC 3.2.1.1), and have also been used [3] for active-site studies of enzymes of the alpha-amylase family. The coupled enzyme α-D-glucosidase (EC 3.2.1.20) acts on the products of the alpha-

0008-6215/95/$09.50
SSDI 0008-6215(95)00216-2

amylases to give nitrophenolate and the sugar moiety. Since the α-D-glucosidase itself hydrolyzes the longer NPαG$_n$, they have been protected from this attack by substitution of their non-reducing ends [2].

Homogenates of *Lactobacillus* sp. no. 26X (Lsp26X) hydrolyzed *p*-nitrophenyl α-maltoside (*p*NPαG$_2$) and α-D-glucopyranoside (*p*NPαG$_1$) at considerable rates. Although an enzyme isolated from the culture broth of *Lactobacillus amylovorus* was reported [4] to be an amylase with an unorthodox substrate specifity, and to hydrolyze *p*NPαG$_1$, in the course of purification [5] of the decycling maltodextrinase from Lsp26X (EC 3.2.1.54; MDase) it became evident that the NPαG$_n$-hydrolyzing activity was not a feature of this enzyme. In this paper, the partial co-purification of the NPαG$_n$-hydrolyzing enzymes, together with the MDase, and their characterization are reported.

2. Experimental

The cultivation of Lsp26X, the preparation of cell homogenates, and anion-exchange chromatography were essentially performed as described [5]. GPC was carried out on Superdex 200 (Pharmacia; column 1.6 × 60 cm, V_t 121 mL; the elution volumes of the column were calibrated using polypeptides with M_r 232,000–12,300), the eluent being 20 mM bis-Tris buffer, pH 6.4, containing 150 mM NaCl. The flow rate was 36 mL/h, and the eluates were collected in 1.8-mL portions. The active fractions were simultaneously concentrated and desalted in a stirred ultrafiltration cell (Amicon), equipped with a Diaflo ultrafiltration membrane PM 10.

Analytical methods.—Protein was determined by the Biuret method [6]. Determination of MDase-activity, the digests of G$_n$, analytical HPLC, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and isoelectric focusing were performed as described [5,7,8]. The M_r of the individual protein bands revealed by SDS-PAGE was calculated according to ref. [9].

The NPαG$_n$-hydrolyzing enzymes were assayed using 10 mM solutions of *p*NPαG$_1$ in 20 mM KH_2PO_4/K_2HPO_4 buffer, pH 6.4, and 3 mM solutions of each *p*NPαG$_2$ and the larger *o*- or *p*NPαG$_n$ in 50 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.3; each solution (1 mL) was incubated (30°C) with an appropriate amount of the enzymes, and the reaction was followed photometrically from the increase in absorbance at 405 nm. The unit of activity (U) was defined as that amount of enzyme which caused the formation of 1 μmol of nitrophenolate per min under the conditions of assay, in which the molar extinction coefficients of nitrophenolate were corrected for the respective pH-value of the digests [10].

The coupled enzymic assays were performed as follows. Mode 1: The reaction was started by addition of MDase (50 mU/mL) or porcine pancreatic alpha-amylase (PPA; 0.8 U/mL). After 4-min pre-incubation (30°C), NPαG$_2$ase (8 mU/mL) was added, and the activity was determined from the increase in absorbance at 405 nm. Mode 2: The reaction was started by the simultaneous addition of MDase (25 mU/mL) or PPA (0.2 U/mL), and NPαG$_2$ase (16 mU/mL); 3 mM substrate solutions, each in 50 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.3, were employed.

Materials.—pNPαG$_1$ and pNPαG$_2$ were purchased from Sigma, and pNPαG$_5$ from Boehringer. pNPαG$_3$, pNPαG$_4$, pNPαG$_6$, oNPαG$_4$, and oNPαG$_6$ were prepared and purified [1] from coupling digests of the cyclodextrin glycosyltransferase (EC 2.4.1.19) from *Klebsiella pneumoniae* M5 A1 with *o*- or pNPαG$_1$ and cyclomaltohexaose. Porcine pancreatic alpha-amylase was purchased from Boehringer. All other substances were commercial materials of high-grade purity.

3. Results

The overall scheme of enzyme purification is shown in Table 1. Chromatography on DEAE-Servacel separated two NPαG$_n$-hydrolyzing enzymes, one of which (preferentially splitting pNPαG$_2$, and designated NPαG$_2$ase) was eluted with 350–480 mM NaCl, thus overlapping the elution area of the MDase (fraction A). The second enzyme (apparently specific for pNPαG$_1$, and designated NPαG$_1$ase) was eluted with 510–730 mM NaCl (fraction B). Chromatography of fraction A on QAE-Sephadex largely separated the NPαG$_2$ase (fraction A$_1$) and the MDase (fraction A$_2$). Further purification

Table 1
Partial copurification of the NPαG$_n$-hydrolyzing enzymes together with the decycling maltodextrinase (MDase) from *Lactobacillus* sp. no. 26X

Step	Volume (mL)	Enzyme	Total activity (U) [a]	Specific activity (U/mg of protein)	Yield (%)	Purification (x-fold)
Cell homogenate [b]	300	NPαG$_2$ase	1760	0.11	100	–
(supernatant)		MDase	5100	0.3	100	–
		NPαG$_1$ase	135	8.8×10^{-3}	100	–
DEAE-Servacel						
A 350–480 [c,d]	8	NPαG$_2$ase	1302	11.5	74	105
		MDase	4250	20.0	83	67
B 510–730	4	NPαG$_1$ase	107	4.0	79	455
QAE-Sephadex						
A$_1$ 520–660	2	NPαG$_2$ase	1021	94.0	58	855
A$_2$ 690–780	2	MDase	3260	100.0	64	333
B 720–800	1	NPαG$_1$ase	80	35.0	59	3980
Superdex 200 [e]						
A$_1$	0.5	NPαG$_2$ase	735	590.0	42	5364
A$_2$	0.5	MDase	3060	121.0	60	403
B (B$_1$) [f]	0.5	NPαG$_1$ase	56	80.0	41	9090

[a] MDase, NPαG$_2$ase, and NPαG$_1$ase were assayed with cyclomaltoheptaose, pNPαG$_2$, and pNPαG$_1$, respectively, as the substrates; for definition of the unit of activity of the NPαG$_n$-hydrolyzing enzymes, see Experimental.
[b] The cultivation of Lsp26X and the preparation of the cell homogenate were performed as described [5].
[c] Elution range (mM NaCl).
[d] Fraction A was chromatographed on QAE–Sephadex to give A$_1$ (NPαG$_2$ase) and A$_2$ (MDase).
[e] Each fraction was separately chromatographed on Superdex 200.
[f] See text.

Table 2
Efficiency of separation of the decycling maltodextrinase (MDase) and the NPαG$_n$-hydrolyzing enzymes by the purification procedure employed

Enzyme	Foreign activities (U/mg of protein) [a]		
	MDase	NPαG$_2$ase	NPαG$_1$ase
MDase	–	0.8	0.1
NPαG$_2$ase	0.5	–	< 0.2
NPαG$_1$ase (B$_1$) [b]	0.1	0.3	–

[a] MDase, NPαG$_2$ase, and NPαG$_1$ase were assayed with cyclomaltoheptaose, *p*NPαG$_2$, and *p*NPαG$_1$ as the substrates.
[b] See text.

of the enzymes was achieved by GPC of the fractions A$_1$, A$_2$, and B on Superdex 200. The efficiency of separation of the MDase and the NPαG$_n$-hydrolyzing enzymes from each other is shown in Table 2.

Evaluation of the molecular data of the enzyme.—NPαG$_2$ase (fraction A$_1$). The GPC-elution volume of maximum activity, matching with the maximum of a smaller peak adjacent to a larger peak (Fig. 1, A$_1$), corresponded to a protein with M_r ~ 163,000. SDS-PAGE revealed one main band (Fig. 2, line 1) with M_r 41,500 (±500). Hence, the M_r 41,500 polypeptide was the subunit of the NPαG$_2$ase, which is thus a homotetramer with M_r 163,000 (±900). Scanning of the SDS-PAGE gel showed that the enzyme was obtained with a purity of 82%.

MDase (fraction A$_2$). The GPC-elution volume of peak maximum was that of a protein with M_r 62,900 (±700), which agreed well with the SDS-PAGE-derived M_r 62,400 (±400). The enzyme was obtained with 96% purity (Fig. 2, line 4).

NPαG$_1$ase (fraction B). The GPC-elution volume of maximum activity corresponded to a protein with M_r ~ 51,000. Since the fraction with maximum activity was situated between two peaks (Fig. 1, B), the active fractions were collected in two portions, B$_1$ (containing 68% of total activity) and B$_2$. SDS-PAGE revealed a series of protein bands (Fig. 2, lines 2 and 3). The elution volume of maximum activity and the protein band intensity on the SDS-PAGE gels made the polypeptide (ca. 20% of total protein) with M_r 25,200 (±400) a candidate for the NPαG$_1$ase (Fig. 2, lines 2 and 3, designated +). Hence, the NPαG$_1$ase might be a homodimer with M_r 51,000 (±600).

Characterization of the NPαG$_n$-hydrolyzing enzymes.—Some characteristics of the enzymes are listed in Table 3. The NPαG$_2$ase (the activity of which depended on the ionic strength of the buffer, and was highest using 50 mM concentrations) exhibited a strong preference for *p*NPαG$_2$ and (to a lesser extent) for *p*NPαG$_3$ (Tables 3 and 4). *p*NPαG$_1$ was scarcely hydrolyzed, and the activities markedly decreased with increasing length of the *o*- or *p*-nitrophenylated substrates. As revealed by HPLC, the enzyme exclusively split the bond between the nitrophenyl group and the sugar moiety, but failed to hydrolyze glycosidic linkages under the conditions of assay. Only small amounts of glucose (< 5% of total carbohydrate) were released from maltose (G$_2$), maltotriose (G$_3$), and maltotetraose (G$_4$) by the NPαG$_2$ase, even when employing high concentrations of enzyme and prolonged incubation.

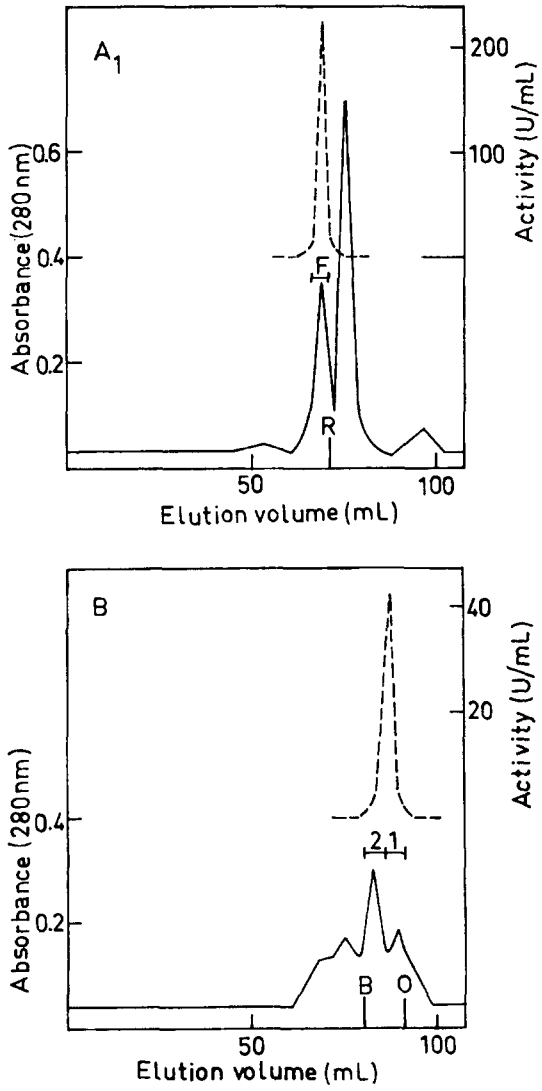

Fig. 1. Gel permeation chromatography of the NPαG$_2$ase (fraction A$_1$) and the NPαG$_1$ase (fraction B) from *Lactobacillus* sp. no. 26X. F and R in A$_1$ indicate the fractions which were pooled for SDS-PAGE analysis and the elution volume of rabbit muscle aldolase (EC 4.1.2.13) with M_r of 158,000, respectively; 1 and 2 in B indicate the areas of active fractions, which were collected separately to give B$_1$ and B$_2$; B and 0 are the elution volumes of bovine serum albumin (M_r 67,000) and of ovalbumin (M_r 43,000). For explanation see text.

The NPαG$_1$ase solely acted on *p*NPαG$_1$ (the low activity observed with *p*NPαG$_2$ was due to the NPαG$_2$ase-impurity in the enzyme, Table 4).

In order to prove the usefulness of the Lsp26X-enzymes for coupled enzymic assays for the MDase and alpha-amylases with NPαG$_n$ as the substrates, two modes of assay

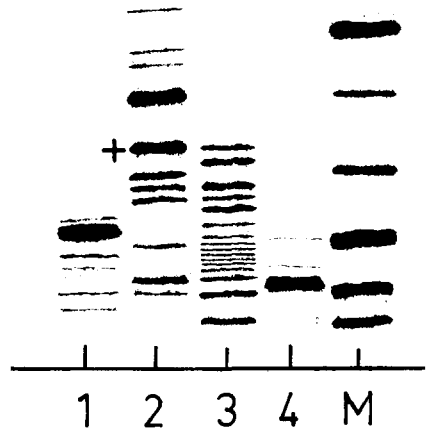

Fig. 2. SDS-PAGE of the NPαG$_n$-hydrolyzing enzymes (NPαG$_2$ase, NPαG$_1$ase) and the decycling maltodextrinase (MDase) from *Lactobacillus* sp. no. 26X. Each 6 μg of protein was applied to gel electrophoresis. Line 1, NPαG$_2$ase; line 2, NPαG$_1$ase (fraction B$_1$); line 3, NPαG$_1$ase (fraction B$_2$, see text); line 4, MDase; line M, the set of marker polypeptides was (from bottom to top) phosphorylase B (M_r 94,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), carboanhydrase B (M_r 30,000), trypsin inhibitor (M_r 20,100), α-lactalbumin (M_r 14,000).

Table 3
Some characteristics of the NPαG$_n$-hydrolyzing enzymes from *Lactobacillus* sp. no. 26X

Characteristics	NPαG$_2$ase	NPαG$_1$ase	Derived from
M_r	163,000 (±900)	51,000 (±600) [a]	GPC (Superdex 200)
Quaternary structure	Homotetramer [4 subunits, M_r 41,500 (±500)]	Homodimer [2 subunits, M_r 25,200 (±400)]	SDS-PAGE
pI	4.1 (±0.06)	n.d.	Isolelectric focusing
Thermostability	None	None	65°C, 10 min
Activation (Mg^{2+}, Mn^{2+})	None	None	Hydrolysis rates in presence of 10^{-6} M $Mg(OAc)_2$ or $Mn(OAc)_2$
pH Stability	4.8–7.6	4.9–7.5	30°C, 6 h
pH Optimum of activity	7.3	6.4	
Specific activities (U/mg of protein)	720 (±15) [b]	80 (±4) [c]	
Michaelis parameters [d]			pNPαG$_2$ and pNPαG$_1$ being the substrates
V (U/mg of protein)	840 (±20) [b]	108 (±6) [c]	
K_m (M)	$9.3 (\pm 0.2) \times 10^{-5}$	$6.3 (\pm 0.2) \times 10^{-3}$	

[a] The data obtained from GPC and SDS-PAGE made this protein a candidate for the NPαG$_1$ase.
[b] Calculated for the pure enzyme.
[c] v_0 and V are those of the enzyme of the actual grade of purity.
[d] Double reciprocal $1/v_0$ vs. $1/[S]$ plots [11] were used to evaluate the Michaelis parameters.

Table 4
Substrate specificity of the NPαG$_n$-hydrolyzing enzymes from *Lactobacillus* sp. no. 26X

Substrate [a]	Activity (U/mg of protein)	
	NPαG$_2$ase	NPαG$_1$ase
pNPαG$_1$	1.24 (0.2) [b]	80 (100) [b]
pNPαG$_2$	590.0 (100)	0.3 (0.4)
pNPαG$_3$	403.7 (68.4)	0
pNPαG$_4$	62.1 (10.5)	0
pNPαG$_5$	24.8 (4.2)	0
pNPαG$_6$	21.7 (3.7)	0
oNPαG$_4$	55.9 (9.5)	0
oNPαG$_6$	15.5 (2.6)	0

[a] Substrate solutions (3 mM) were incubated with 8 or 16 mU/mL of the NPαG$_2$ase and 30 mU/mL of the NPαG$_1$ase separately.
[b] In parentheses: % of activity. The maximum activities observed with pNPαG$_2$ (NPαG$_2$ase) and with pNPαG$_1$ (NPαG$_1$ase) were set at 100%.

were carried out. It was evident that the actions of both MDase and PPA delivered the preferential substrates for the NPαG$_2$ase (Table 5; for detailed discussion see the next section).

Table 5
Determination of enzymic activities observed with larger NPαG$_n$ in coupled assays with NPαG$_2$ase and the decycling maltodextrinase (MDase) or alpha-amylase (PPA)

Assay 1 [a]	Enzymic activities (U/mg of protein)			
Substrate	MDase [b]	+NPαG$_2$ase	PPA	+NPαG$_2$ase
pNPαG$_4$	0.04	530	0	301
pNPαG$_5$	0.016	366	0	314
pNPαG$_6$	0.016	317	0	320
oNPαG$_4$	0.04	512	0	276
oNPαG$_6$	0.01	295	0	307
Assay 2 [c]	Enzymic activities (U/mg of protein)			
	NPαG$_2$ase	+MDase		+PPA
pNPαG$_4$	20	127		76
pNPαG$_5$	8	128		84
pNPαG$_6$	7	122		92
oNPαG$_4$	18	130		68
oNPαG$_6$	5	120		86

[a] The activities are related to the NPαG$_2$ase.
[b] NPαG$_2$ase impurity of the MDase.
[c] The activities are related to the MDase and PPA (the activity of the MDase with cyclomaltoheptaose was 121 U/mg, and that of PPA with soluble starch ~1000 U/mg). The activities were corrected for those observed for the NPαG$_2$ase with the substrates.

The coupled assay of MDase/NPαG$_1$ase with pNPαG$_3$ gave an MDase-activity of 17 U/mg, corresponding to 25% of the activity observed with G$_3$ [5], i.e., pNPαG$_1$ was among the hydrolysis products. However, the coupled assays of MDase or PPA + NPαG$_1$ase with the longer substrates did not indicate the formation of NPαG$_1$.

4. Discussion

Lsp26X produces two NPαG$_n$-hydrolyzing enzymes, the features of which differ significantly from α-D-glucosidases, and from a pNPαG$_2$-hydrolyzing enzyme isolated from a strain of *Klebsiella pneumoniae* [10]. In comparison with the β-D-galactosidase (EC 3.2.1.23), which is known to hydrolyze oNPβGal and pNPβGal 7 and 17 times faster, respectively, than lactose [12], the preference of the Lsp26X-enzymes for the nitrophenylated substrates was markedly more pronounced.

The NPαG$_2$ase exhibited a high affinity for NPαG$_2$ (Table 3) which allowed the enzymic determination of very low concentrations of this compound. Accordingly, this feature made the enzyme highly attractive for coupled enzymic assays for MDase or alpha-amylases. The Mode 1 employed (Table 5) served for assaying the reliability of the coupled test, because it gave the concentrations of the products (NPαG$_2$, NPαG$_3$) formed by the action of MDase or PPA on the longer NPαG$_n$ in the course of pre-incubation. Previous analyses revealed that G$_2$ is the main product formed by the action of the Lsp26X-MDase on G$_n$ or cyclomaltosaccharides [5]. Hence, releasing G$_2$ from NPαG$_4$ and NPαG$_5$ resulted in the formation of NPαG$_2$ and NPαG$_3$, respectively. About 90% of the NPαG$_2$ase-activity determined with 3 mM pNPαG$_2$ and pNPαG$_3$ was observed, i.e., ca. 0.4 μmol/mL of substrate was hydrolyzed (derived from the substrate saturation curve of the NPαG$_2$ase). Assuming stepwise hydrolysis of G$_2$-units from the non-reducing end, the concentration of NPαG$_2$ must be lower with NPαG$_6$ under the conditions of assay. Indeed, only $\sim$ 53% of maximum activity was found, corresponding to a concentration of 0.1 μmol/mL NPαG$_2$. The preferential release of G$_2$ by the MDase was confirmed by the fact that hydrolysis of pNPαG$_3$ delivered the substrate of the NPαG$_1$ase.

Because of the lower activities (see below), less NPαG$_2$/NPαG$_3$ was formed by the action of PPA on the longer NPαG$_n$. Thus, in the digests even with NPαG$_6$, only 54% of maximum NPαG$_2$ase activity was observed.

Mode 2 of the coupled assay was thought to determine the activities of MDase or PPA. Since the concentrations of NPαG$_2$/NPαG$_3$ were lower, they must be rate-limiting for the NPαG$_2$ase. The MDase-assays gave comparable activities with NPαG$_4$ and NPαG$_5$, and again somewhat lower with NPαG$_6$, corresponding to 55% (NPαG$_4$), 50% (NPαG$_5$), and 41% (NPαG$_6$) of the activities observed with G$_4$–G$_6$ [5]. The assays with PPA confirmed that the hydrolysis rates increased with increasing length. However, the activity found with the most suitable substrate, NPαG$_6$, corresponded to only 9% of that determined with soluble starch, indicating that the NPαG$_n$ generally are poorer substrates for this enzyme. Scaling-up the conditions towards the needs of alpha-amylases should improve the assay, i.e., the NPαG$_2$ase will be of value for a coupled enzymic test for these enzymes.

Acknowledgement

G. Laule is thanked for his help in performing the GPC-runs, the SDS-PAGE, and isoelectric focusing.

References

[1] K. Wallenfels, B. Meltzer, G. Laule, and G. Janatsch, *Fresenius Z. Anal. Chem.*, 301 (1980) 169–170.
[2] K. Omichi and T. Ikenaka, *J. Biochem. (Tokyo)*, 97 (1985) 977–982.
[3] S. Abe, Y. Nagamine, K. Omichi, and T. Ikenaka, *J. Biochem. (Tokyo)*, 110 (1991) 756–761.
[4] A. Burgess-Cassler, in A.R. Hedges (Ed.), *Minutes of the Sixth International Symposium on Cyclodextrins, Chicago, April 21–24*, Editions de Santé, Paris, 1992, pp 377–399.
[5] H. Bender, *Appl. Microbiol. Biotechnol.*, (1995), in press.
[6] G. Beisenherz, H.J. Boltze, T. Bücher, R. Czok, K.H. Garbade, E. Meyer-Arendt, and G. Pfleiderer, *Z. Naturforsch., Teil B*, 8 (1953) 555–577.
[7] H. Bender, *Appl. Microbiol. Biotechnol.*, 39 (1993) 714–719.
[8] H. Bender, *Carbohydr. Res.*, 263 (1994) 123–135.
[9] K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406–4412.
[10] P. Földi, Ph.D. Thesis, Universität Freiburg i. Br., 1977.
[11] H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, 56 (1934) 658–666.
[12] K. Wallenfels, M.L. Zarnitz, G. Laule, H. Bender, and M. Keser, *Biochem. Z.*, 331 (1959) 459–485.