Carbohydrate Research, 209 (1991) 145–153
Elsevier Science Publishers B.V., Amsterdam

On the role of histidine residues in cyclodextrin glycosyltransferase: chemical modification with diethyl pyrocarbonate

Hans Bender
Institut für Organische Chemie und Biochemie der Universität Freiburg i. Br., Albertstr. 21, D-7800 Freiburg i. Br. (F.R.G.)

(Received May 17th, 1990; accepted for publication, July 5th, 1990)

ABSTRACT

Ethoxyformylation with diethyl pyrocarbonate of ~1.5 His residues per molecule of enzyme reduced the cyclising activity of both the α-cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* strain M 5 al and the β-cyclodextrin glycosyltransferase from *Bacillus circulans* strain 8 by >90%. Pre-incubation with substrate protected the enzymes from ethoxyformylation. Digestion of starch by the modified enzymes resulted in a delayed formation of cyclodextrins (cyclomalto-oligosaccharides, CDs), but a marked increase in the production of reducing saccharides. Similarly, coupling of αCD and maltose and successive disproportionation yielded mainly glucose and malto-oligosaccharides. The results are discussed in the context of the role of conserved His residues for binding of substrate and the transfer reactions.

INTRODUCTION

Comparison of the primary structures of cyclodextrin glycosyltransferases {(1→4)-α-D-glucan[(1→4)-α-D-glucopyranosyl]transferase (cyclising), EC 2.4.1.19; CGTase} with those of alpha-amylases {(1→4)-α-D-glucan[glucano]hydrolase, EC 3.2.1.1} revealed similarities in sequence at least for four conserved regions, including the catalytically active amino acid residues and the residues responsible for the binding of substrate and calcium of the alpha-amylases[1]. The three-dimensional structure of the β-CGTase from *Bacillus circulans* strain 8 (Bc-β-CGTase) has been elucidated[2] at a resolution of 3.4 Å. The chain fold has been subdivided into five domains that could be considered as an alpha-amylase fold[3,4] with two additional domains.

The mechanism of the reversible (1→4)-α-D-glucopyranosyltransfer reactions is still not clear. The C-terminal regions show high homology between all CGTases and have been supposed to be involved in catalysis and in the binding of substrate[5]. However, deletion of the 90 C-terminal residues of the α-CGTase from *Klebsiella pneumoniae* strain M 5 al (Kp-α-CGTase) did not alter the catalytic properties[6]. Essentially, the differences between alpha-amylases and CGTases might be restricted to the N-terminal domains.

Before starting genetic engineering experiments, the role of functional amino acid residues for the transfer reactions was investigated by selective chemical modification.

0008-6215/91/$03.50

Modification of CGTases with *N*-bromosuccinimide revealed that tryptophan residues participate in the binding of the substrate, but only slightly in catalysis[7]. Histidine (His) residues of alpha-amylases[3] also contribute to binding of substrate and His-122 (region 1), His-210 (region 2), and His-296 (region 4) are subsites of Taka-amylase A. The CGTases contain conserved His residues in corresponding positions.

Diethyl pyrocarbonate (ethoxyformic anhydride) selectively ethoxyformylates His residues in proteins[8–13], and the effect of ethoxyformylation of Kp-α-CGTase and Bc-β-CGTase on the cyclisation reaction is now reported.

EXPERIMENTAL

Enzymes. — Crude Kp-α-CGTase was kindly provided by Dr. G. Schmid (Consortium für Elektrochemische Industrie, Munich). Recombinant Bc-β-CGTase was isolated[1] from the clone *Escherichia coli* pBC22. The enzymes were purified by affinity chromatography[2,6] on β-CD-Sepharose 6B. Sodium dodecylsulfate–polyacrylamide gel electrophoresis revealed one protein band for each enzyme preparation.

Substrates. — Soluble starch was obtained from Merck, and malto-octaose (G_8) was prepared as described[6].

Modification of the enzymes. — 0.15–0.4mM Solutions of the CGTases in 20mM Tris-HCl buffer (pH 7.0), which contained 3mM $CaCl_2$ and 0.4–6.0mM diethyl pyrocarbonate (Serva, 0.3M in dry ethanol), were incubated for 1 h at 25°. Reaction with the His residues resulted[9,10] in a change in the u.v. spectra at 240 nm, from the intensity of which the number of His residues ethoxyformylated was estimated, using an absorption coefficient[9] of 3.2×10^3 L.mol^{-1}.cm^{-1}. In parallel, the enzymes were each pre-incubated (4°, 5 min) with aqueous 1.5% soluble starch, before treatment with diethyl pyrocarbonate.

Analytical methods. — Total carbohydrate was determined with the anthrone reagent[14], reducing end-groups with the Nelson reagent[15], and protein by the biuret method[16].

The initial rates of cyclisation were followed by an optical assay[6] using G_8 (0.07–5.0mM) and soluble starch (0.08–1.7 g/L) as the substrates. The specific activity is expressed as kat.kg^{-1} of protein, and the molar catalytic activity $V/[E_o]$, where V and $[E_o]$ are the maximum velocity of cyclisation and the molar concentration of the enzyme, respectively, as kat.mol^{-1}. The Michaelis constants K_m and V were determined[17] from double reciprocal $1/v_o$ *vs.* 1/[S] plots, where v_o and [S] are the initial rate of cyclisation and the concentration of substrate, respectively.

H.p.l.c. — 3% Gelled potato starch in 10mM sodium phosphate buffer (pH 6.8) was incubated (30°) with the CGTases (cyclising activity 0.8–2.8 μkat/g of starch). Aliquots of the digests were withdrawn at intervals, the high-molecular-weight products were precipitated by the addition of methanol to 60%, and the supernatant solutions were concentrated to their original volumes and analysed[6,18] by h.p.l.c. For the analysis of the linear products, the CDs were removed as their complexes with cyclohexane or bromobenzene.

The coupling reaction was followed by incubation (30°) of 30mM α-CD and 9mM maltose in 10mM sodium phosphate buffer (pH 6.8) together with the CGTases (cyclising activity 0.08–0.28 μkat/mmol of substrate). Aliquots of the digests were withdrawn at intervals, and analysed by h.p.l.c.

Amino acid analysis. — Tyr and His residues of the native and ethoxyformylated CGTases were determined essentially as described[19].

RESULTS AND DISCUSSION

Ethoxyformylation of ~1.5 His residues per molecule of enzyme, obtained at molar ratios of diethyl pyrocarbonate to enzyme of 11.2:1 (Kp-α-CGTase) and 20:1 (Bc-β-CGTase), resulted in a >90% loss of cyclising activity (Fig. 1, curves I). Complete inhibition occurred at molar ratios of diethyl pyrocarbonate to enzyme of >20:1, but cleavage of imidazole rings and ethoxyformylation of Tyr residues could not be excluded. However, as judged from the absorption spectra (Table I) and amino acid analysis (Table II) of native and modified proteins, the His residues were ethoxyformylated exclusively at the lower molar ratios.

Pre-incubation with aqueous 1.5% soluble starch protected the CGTases effi-

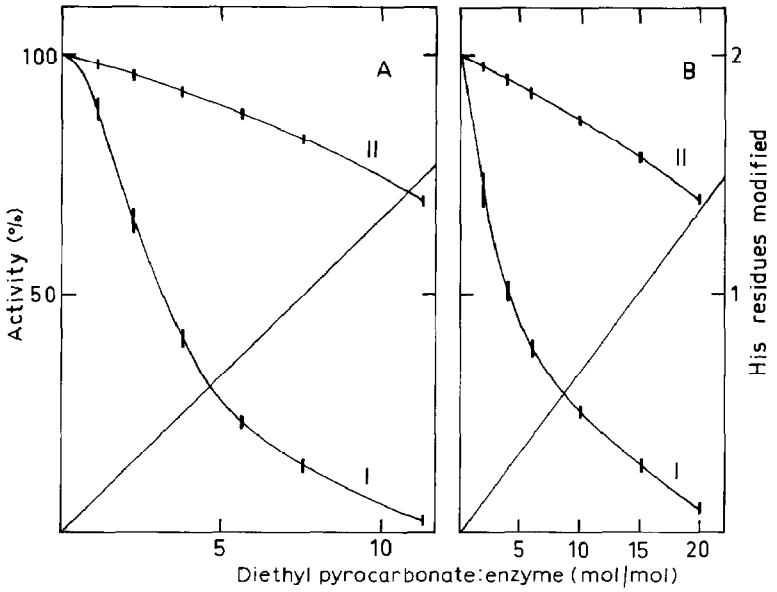

Fig. 1. Effect of ethoxyformylation of the His residues of Kp-α-CGTase (A) and Bc-β-CGTase (B) on the rates of cyclisation (curves I). The initial cyclisation was followed by an optical assay. The concentrations of enzyme were 0.7–3.5 (Kp-α-CGTase) and 1.6–7.3 μg/mL (Bc-β-CGTase), the concentrations of substrate were 1 g/L (soluble starch) and 0.76mM (G_8). The 100% activities of Kp-α-CGTase were 3.35 (soluble starch) and 3.3 kat.kg^{-1} of protein (G_8), and of Bc-β-CGTase 1.03 (soluble starch) and 1.0 kat.kg^{-1} of protein (G_8). The decrease in activity determined with soluble starch and G_8 was of the same order. Curves II, the enzymes were pre-incubated (4°, 5 min) with aqueous 1.5% soluble starch.

TABLE I

Changes in the u.v. spectra by ethoxyformylation of Kp-α-CGTase[a] and Bc-β-CGTase[b]

Enzyme	*Molar ratio DEP[c]/enzyme*	*Spectral change ($\times 10^{-2}$), wavelength (nm)*					*His residues modified[d]*
		240	*260*	*278*	*300*	*320*	
Kp-α-CGTase	1.12	0.28	0.1	0	0	0	0.1
	2.24	0.8	0.26	0	0	0	0.3
	3.75	1.3	0.8	0	0	0	0.49
	7.5	2.61	1.4	0	0	0	1.0
	11.2	3.95	2.1	0	0	0	1.5
Bc-β-CGTase	2.0	0.32	0.12	0	0	0	0.13
	4.0	0.65	0.21	0	0	0	0.26
	6.0	1.0	0.6	0	0	0	0.4
	10.0	1.6	0.9	0	0	0	0.65
	15.0	2.5	1.1	0	0	0	1.01
	20.0	3.6	1.9	0	0	0	1.46

[a,b] The concentrations of the CGTases were 8.23 and 7.7 $\times 10^{-6}$M, respectively. [c] Diethyl pyrocarbonate. [d] The number of ethoxyformylated His residues was estimated from changes at 240 nm, using an absorption coefficient of 3.2 $\times 10^3$ L.mol^{-1}.cm^{-1}.

TABLE II

Amino acid analysis of native and ethoxyformylated Kp-α-CGTase (Kp) and Bc-β-CGTase (Bc)[a]

Amino acid	*Residues per molecule*					
	Native		*Modified*		*From sequence*[b]	
	Kp	*Bc*	*Kp*	*Bc*	*Kp*	*Bc*
Tyr	28.4	32.1	28.6	32.3	28	32
His	10.4	12.2	9.1	11.1	10	12

[a] Modification with 15-fold (Kp-α-CGTase) and 20-fold (Bc-β-CGTase) excess of diethyl pyrocarbonate over protein. [b] Refs. 1 and 20.

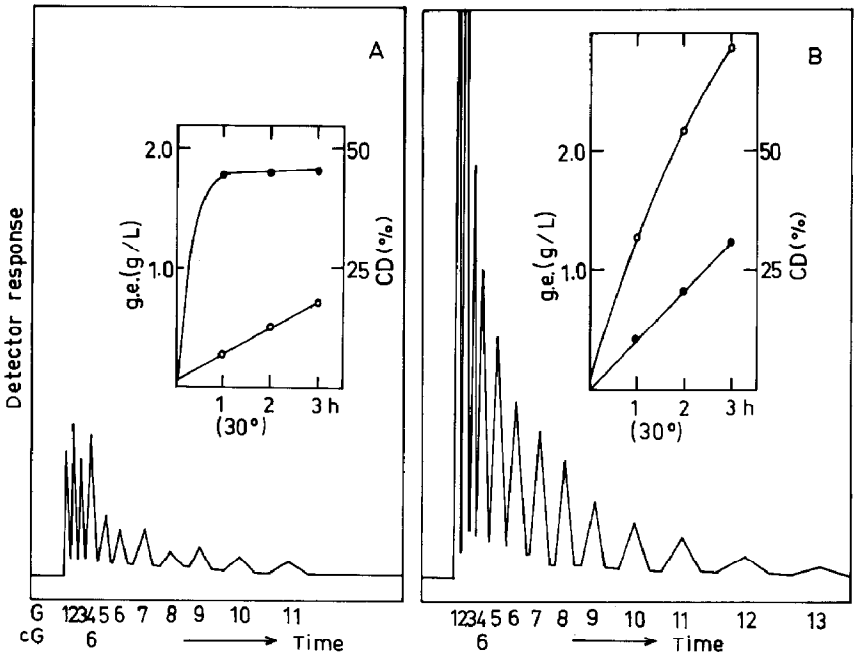

Fig. 2. H.p.l.c. of the linear products (50 μL of each digest injected) from 3% starch digested (30°, 3 h) with native (A) and ethoxyformylated (B) Kp-α-CGTase. Cyclising activities of 2.4 (native) and 0.8 μkat (modified) enzyme per g of starch were employed. Before analysis of the linear products, the CDs were removed as their complexes with cyclohexane or bromobenzene. Since the solubility of the α-CD–cyclohexane complex is 1.5 g/L (20°), some of it remained in the fraction of the saccharides, and was eluted near to maltotetraose. G_n, malto-oligosaccharide; cG_n, CD with n glucose residues. For preparation of the digests and their analysis, see Experimental. Inserts: time-dependent increase in CDs (—●—, % of total carbohydrate) and glucose equiv. (g.e., —○—).

TABLE III

Effect of ethoxyformylation on the molar catalytic activity ($V/[E_o]$) and the Michaelis constant (K_m) determined for the cyclisation reaction[a] of Kp-α-CGTase (Kp) and Bc-β-CGTase (Bc)

CGTase	*Substrate*	*Native enzyme*[b]		*Ethoxyformylated enzyme*[c]	
		$V/[E_o]$	K_m	$V/[E_o]$	K_m
Kp	G_8	230 (±3.1)[d]	2.5×10^{-4} M (±0.2)	11 (±1.2)	3.3×10^{-4} M (±0.3)
	Soluble starch	231 (±1.9)	0.286 g/L (±0.06)	13 (±1.8)	0.5 g/L (±0.08)
Bc	G_8	75 (±2.2)	1.73×10^{-4} M (±0.2)	7 (±1.2)	2.4×10^{-4} M (±0.2)
	Soluble starch	71 (±1.8)	0.163 g/L (±0.03)	6 (±1.4)	0.25 g/L (±0.02)

[a] The rate parameters were determined from double reciprocal $1/v_o$ *vs.* 1/[S] plots. [b] The concentrations were 0.7 (Kp-α-CGTase) and 1.6 μg/mL (Bc-β-CGTase), respectively. [c] The concentrations were 3.5 (Kp-α-CGTase) and 7.3 μg/mL (Bc-β-CGTase). ~1.5 His residues per molecule of enzyme were modified. [d] Standard deviations (ref. 21).

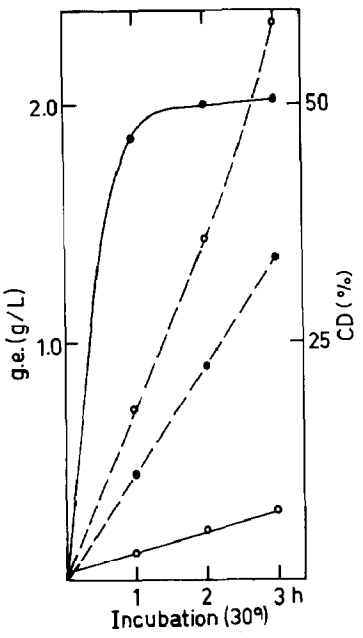

Fig. 3. Time-dependent increase in CDs (–●–, % of total carbohydrate) and glucose equiv. (g.e., –○–) by digestion (30°) of 3% starch with native (—) and ethoxyformylated (– –) Bc-β-CGTase. Cyclising activities of 2.8 (native) and 0.9 μkat (modified enzyme) per g of starch were employed. For preparation of the digests and their analysis, see Experimental.

ciently from reaction with diethyl pyrocarbonate (Fig. 1, curves II) and ~70% of the activity was retained even at the highest molar ratios of diethyl pyrocarbonate to enzyme employed, indicating a tight binding of the substrate to the essential His residues. Ethoxyformylation of the CGTases caused a decrease in the affinity for the substrates (Table III).

Digestion of starch with the modified enzymes (15-fold excess of diethyl pyrocarbonate over protein) resulted in a delayed formation of CDs but a marked enhancement of the production of reducing saccharides (Figs. 2 and 3; except for the ratios of CDs, Bc-β-CGTase behaved similarly; h.p.l.c. data not reported). Likewise, coupling assays[18,22] revealed that incubation of αCD and maltose with Kp-α-CGTase for 3 h yielded 70% of total carbohydrate as CDs (α,β,λ-ratios 1:1.3:0.21) and 30% of malto-oligosaccharides (Fig. 4A), whereas coupling and disproportionation with the ethoxyformylated CGTase yielded mainly glucose and malto-oligosaccharides, but only 24% of CDs (mainly αCD) [Fig. 4B; the Bc-β-CGTase showed similar behaviour (data not reported)]. Thus, the modified CGTases had lost most of the cyclising activity but retained the ability to form linear products. From the profile of the products, an altered disproportionation behaviour rather than hydrolysis occurred.

Therefore, His residues are involved in the cyclisation reaction. Kp-α-CGTase and Bc-β-CGTase contain 10 and 12 His residues, respectively, most of which are situated in the N-terminal regions. Of the His residues, 7 are conserved in CGTases, 5 in CGTases and Taka-amylase A (Table IV; italicised numbers), and 3 are subsites of the

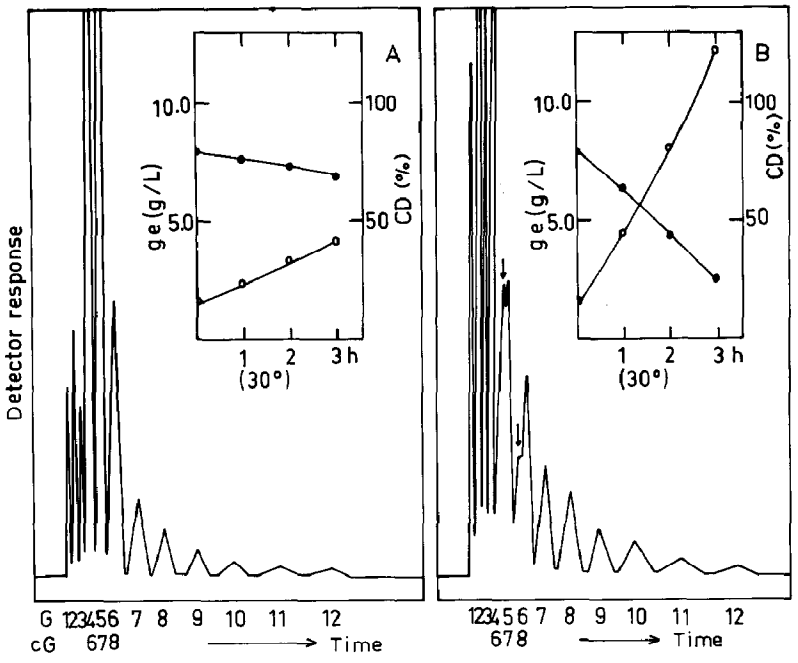

Fig. 4. H.p.l.c. of the products (20 μL of each digest injected) from coupling and disproportionation (30°, 3 h) of 30 mM αCD and 9mM maltose with native (A) and ethoxyformylated (B) Kp-α-CGTase and (inserts) time-dependent decrease in substrate (–●–, % of total carbohydrate) and increase in glucose equiv. (g.e., –○–). Cyclising activities of 0.24 (native) and 0.08 μkat (modified) enzyme per mmol of substrate were employed. G_n and cG_n, malto-oligosaccharides and CDs with n glucose residues, respectively. The arrows in B indicate βCD and γCD. For preparation of the digests and analysis, see Experimental.

TABLE IV

Position of His residues in Bc-β-CGTase[a] and Kp-α-CGTase[b], compared to those in Taka-amylase A (TAA)[c]

Region	*Bc-β-CGTase*	*Kp-α-CGTase*	*TAA*
	98[d]	*91*	-
	-	104	-
	126	*119*	*108*
1	*140*	*135*	*122*
	176	*169*	*147*
	177	*170*	-
	-	186	-
	202	-	-
2	*233*	*227*	*210*
	249	-	-
4	*327*	*332*	*296*
	-	-	334
	-	390	-
	502	-	
	630	-	
	667	-	

[a] 684 Residues (ref. 1). [b] 625 Residues (ref. 20). [c] 478 Residues (ref. 23). [d] The numbers in italics indicate the residues conserved.

amylase (regions 1,2, and 4), and presumedly also of the CGTases, because of their conserved positions. Thus, the refinement of the Bc-β-CGTase structure revealed that His-327 is situated[7] at the active site opposite to the putative catalytic site Asp-229. Since the catalytic sites are supposed[6] to be situated between subsites 3 and 4 (Kp-α-CGTase) and 2 and 3 (Bc-β-CGTase), respectively, His-332 of Kp-α-CGTase and His-327 of Bc-β-CGTase should function as one of these subsites. Accordingly, the loss of binding affinity caused by ethoxyformation of these His residues may result in an altered binding mode of substrate that is not suited for cyclisation, but allows disproportionation to yield glucose, maltose, and maltotriose as the main products.

It is noteworthy that 2 His residues are found in a characteristic stretch of the CGTases, that is only partly conserved in Taka-amylase A:

Kp-α-CGTase 166-Gly-Trp-Tyr-His-His-Asn-Gly-Gly-
Bc-β-CGTase 174-Gly-Tyr-Phe-His-His-Asn-Gly-Gly-
Taka-amylase A 144-Asp-Tyr-Phe-His-Pro-Phe-Cys-Phe-

The position of His-177 and His-178 near the active site of Bc-β-CGTase suggests that they could be important for catalysis.

ACKNOWLEDGMENTS

Dr. G. Schmid is thanked for the provision of crude Kp-α-CGTase, and Dr. E. Schiltz for the performance of the amino acid analyses.

REFERENCES

1 L. Nitschke, K. Heeger, H. Bender, and G. E. Schulz, *Appl. Microbiol.*, (1990), 33 (1990) 542–546.
2 B. E. Hofmann, H. Bender, and G. E. Schultz, *J. Mol. Biol.*, 209 (1989) 793–800.
3 Y. Matsuura, M. Kusunoki, W. Harada, and M. Kakudo, *J. Biochem. (Tokyo)*, 95 (1984) 697–702.
4 G. Buisson, R. Haser, E. Duée, and F. Payan, *EMBO J.*, 6 (1987) 3909–3916.
5 K. Kimura, S. Kataoka, A. Nakamara, T. Takano, S. Kobayashi, and K. Yamane, *Biochem. Biophys. Res. Commun.*, 161 (1989) 1273–1279.
6 H. Bender, *Carbohydr. Res.*, 206 (1990) 257–267.
7 H. Bender, unpublished results.
8 A. Muhlrád, G. Hegyi, and G. Tóth, *Acta Biochim. Biophys. Acad. Sci. Hung.*, 2 (1967) 19–29.
9 J. Ovàdi, S. Libor, and P. Elodi, *Acta Biochim. Biophys. Acad. Sci. Hung.*, 2 (1967) 455–458.
10 W. B. Melchior and D. Fahrney, *Biochemistry*, 9 (1970) 251–258.
11 L. G. Dann and H. G. Britton, *Biochem. J.*, 131 (1974) 729–738.
12 M. J. Loosemore and R. F. Pratt, *FEBS Lett.*, 72 (1976) 155–158.
13 U. Bagert and K. H. Röhm, *Biochim. Biophys. Acta*, 999 (1989) 36–41.
14 F. J. Viles and L. Silverman, *Anal. Chem.*, 21 (1949) 950–953.
15 N. Nelson, *J. Biol. Chem.*, 153 (1944) 375–380.
16 G. Beisenherz, H. J. Boltze, T. Bücher, T. Czok, K. H. Garbade, E. Meyer-Arendt, and G. Pfleiderer, *Z. Naturforsch. Teil B*, 8 (1953) 555–577.
17 H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, 56 (1934) 658–666.
18 H. Bender, *Carbohydr. Res.*, 117 (1983) 1–11.
19 R. L. Heinrikson and S. C. Meredith, *Anal. Biochem.*, 136 (1984) 65–74.
20 F. Binder, O. Huber, and A. Böck, *Gene*, 47 (1986) 269–277.
21 W. L. Masterton and E. J. Slowinski, *Mathematical Preparations for General Chemistry*, Verlag Chemie, Weinheim, 1983.
22 H. Bender, *Carbohydr. Res.*, 65 (1978) 85–97.
23 E. A. MacGregor and B. Svensson, *Biochem. J.*, 259 (1989) 145–152.