

The Michaelis constants ratio for two substrates with a series of fungal (mould and yeast) β -galactosidases

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Abstract

A comparative analysis of kinetic data for fungal β -galactosidases has revealed that the ratio of Michaelis constants (MCR) with two substrates (lactose and *o/p*-nitrophenyl- β -D-galactopyranoside, NPG) is approximately constant for certain groups of enzymes. It equals 35 ± 3 and 10 ± 1.5 for the mould and the yeast enzymes, respectively.

$$\text{MCR} = \frac{K_M(\text{lactose})}{K_M(\text{NPG})}$$

Thus, MCR provides a simple and fast method for identification of related enzymes. MCR for a group of enzymes with a pair of standard substrates may serve as an identification number of this group. A substantial deviation of MCR for a particular enzyme from the group parameter might be an indication of possible errors in K_M determination, or in assignment of the enzyme to this group. An important advantage of this approach is that the experimental K_M values may be obtained for enzymes with different purity and concentration.

Within the framework of classical Michaelis–Menten kinetics and in accordance with the customary use of K_M for a comparison of enzyme–substrate affinity, the MCR parameter may be interpreted as a relative substrate affinity. This proportion remains nearly the same for different mould (or yeast) β -galactosidases, though the affinity to a particular substrate may vary essentially from enzyme to enzyme. The constancy of MCR may be a manifestation of structural similarity of binding sites for these enzymes.

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1. Introduction

When an enzyme catalyzes reactions of several substrates, the evaluation of its relative affinity for these substrates is of importance. A few parameters combining the Michaelis–Menten constant (K_M), the maximum initial velocity (V_{\max}) or other parameters have been designed for this purpose: selectivity constant, $(V_{\max 2}/V_{\max 1})(K_{M2}/K_{M1})$ [1], specificity constant, k_{cat}/K_M [2,3], maximum proteolytic coefficient, $k_2/2.3K_M$ [4,5], phosphorylation coefficient [6]—generalized later as a catalytic coefficient [7].

The last one, $C_{\text{cat}} = (V_{\max 2}/K_{M2})/(V_{\max 1}/K_{M1})$, has been suggested as a parameter for “the relative availability” of two substrates” [7]. However, it is well known that V_{\max} is not a fundamental characteristic of enzyme, because it depends on the enzyme purity and concentration [2,3,8–11]. The Michaelis–Menten constant, K_M , is a more fundamental parameter. Although, K_M is not a dissociation constant [10] (however, see discussions in [4,5,12–14]), it is often used as an “affinity parameter”, in particular for a comparison of enzyme affinity to various substrates: the smaller is K_M , the larger is believed to be the affinity [1,4,5,11–31]. Thus, K_M is customarily considered to be roughly reciprocal to the enzyme–substrate affinity, which follows from the steady-state derivation of K_M in classical Michaelis–Menten kinetics as the sum of the constants for breakdown of

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enzyme–substrate complex, k_{-1} and k_2 , divided by the constant for its formation, k_1 [2,3,8,10].

Working on the accurate (re)measuring of Michaelis constant for fungal β -galactosidases from *Aspergillus oryzae*, *Curvularia inaequalis*, *Penicillium canescens* and *Penicillium multicolor* [32,33], one of us (NMS) had noticed that, in spite of substantially different K_M values for these three enzymes, the ratio $K_M(\text{lactose})/K_M(\text{NPG})$ was always approximately the same and fell in the range 33–36. The available data for a few other fungal β -galactosidases also supported this observation, though with a couple of exceptions [32,33].

In this study we have performed a systematic comparative analysis of kinetic parameters and enzymatic properties for a set of fungal (mould and yeast [34]) β -galactosidases (lactases, β -D-galactoside galactohydrolases, EC 3.2.1.23), the enzymes that hydrolyze β -1,4-D-galactosidic bonds, such as the one present in lactose. The previously reported regularity [32,33,35–38] has been confirmed and expanded. Substantially different MCR values have been found for the mould and the yeast enzymes. We suggest herewith the *Michaelis constants ratio* (MCR, Eq. (1)) as a prospective tool for enzyme classification.

$$\text{MCR}_{(S_2/S_1)} = \frac{[1/K_M(S_1)]}{[1/K_M(S_2)]} = \frac{K_M(S_2)}{K_M(S_1)} \quad (1)$$

Considering the value $1/K_M$ as a conventional measure of enzyme–substrate affinity, MCR can be used as an approximate characteristic for the relative affinity of enzymes towards two substrates. The constancy of MCR along with similarities in enzymatic properties for a group of enzymes may be an indication of structural similarity of their binding sites.

2. Data evaluation

We collected and compared the literature data on kinetic parameters, enzymatic properties, and amino acid composition for a series of 35 fungal β -galactosidases with steady-state kinetics obeying the Michaelis–Menten relationship (Tables 1–3). To the best of our knowledge, such an extensive compilation of the kinetic parameters for fungal β -galactosidases has never been published before (Table 1 covers the data from 1952 through June 2004).

Wide variations of the apparent K_M values have been reported for the most frequently studied enzymes: *Aspergillus niger*, *A. oryzae*, *Kluyveromyces (Saccharomyces) lactis* and *Kluyveromyces (Saccharomyces) fragilis* (Table 1). The estimations of K_M values may be affected by many factors that include, but are not limited to: differences in enzyme producing strains (e.g., compare entries 69 versus 71 in Table 1), in experimental conditions (temperature, pH, substrate and product concentrations, etc.) [13,14,26,39–42], difference in a nature of measured product (e.g. glucose or galactose [23,43,44]), difference in methods of measurement

(e.g. for lactose hydrolysis: the glucose oxidase–peroxidase method [45–47], NAD^+ reduction [23,44], HPAEC [44], or electrophoretically mediated microanalysis [48], etc.), and differences in methods of K_M calculation (see below). For example, a K_M value of 12.2 ± 1.9 mM was obtained when galactose formation was measured in lactose hydrolysis by β -galactosidase from *K. lactis* [43]. When glucose formation was measured in the same reaction, a K_M value of 16.8 ± 0.1 mM was found [43].

Besides, the growth conditions of enzyme producing microorganism can be of importance. Thus, K_M for β -galactosidase from *K. lactis* was lower when a cell growth medium contained 15% lactose [49]. K_M was larger for *Kluyveromyces marxianus* enzyme, when the cells were grown at 45°C [50] compared to 30°C [51].

It had been noted that K_M for *A. oryzae* β -galactosidase with lactose ranged from 34 to 69 mM depending on how it was calculated [20]. Similar, though smaller variations of K_M were found for the enzymes from *A. niger* and *K. lactis* when four graphical methods were compared [52]. There are few determinations of kinetic parameters for fungal β -galactosidases by non-linear regression methods (Table 1, entries 16, 18, 23, 38, 73, 78, 84, 95, 96, 98). Most published kinetic parameters were determined by one or more of several graphical methods: Lineweaver–Burk (double-reciprocal) plot [11,12,15], Hanes–Woelf plot [11,53,54], Eadie–Hofstee plot [11,55], and Cornish–Bowden–Eisenthal (direct linear) plot [3,56,57]. The Lineweaver–Burk plot is by far the most widely used, although it was shown to be statistically the least reliable (for detailed discussions see [3,11,52,53,55–61]). The major flaw of this method is the disproportional susceptibility to experimental errors in the range of low concentration of substrate. Unfortunately, the measurements at low concentrations played a substantial role in some estimations [19,44,48,62–67] (see Table 1, entries 3, 4, 15, 20, 21, 31, 45, 50, 51, 64; this identification could be done only when a plot or a concentration range was published). We roughly estimated a significance of such errors by application of Hanes–Woelf and direct linear plots to the data extracted from Lineweaver–Burk plot for *Aspergillus foetidus* β -galactosidase [66] (unfortunately, the numerical experimental data are never published): the K_M value with lactose was recalculated as 61 mM instead of published 68.8 mM [66]. Additional examples are provided by the differences between K_M values obtained from different linear plots for enzymes from *A. niger*, *A. oryzae*, and *K. lactis* (Table 1, entries 19, 33, and 75, 79, respectively).

We have to mention that the accuracy of the K_M measurement and reporting is unfortunately not an issue of high priority in studies on enzyme kinetics. Some of the previously published results for enzymes from *A. oryzae*, *C. inaequalis*, *P. canescens* and *P. multicolor* have been shown to be substantially inaccurate by a thorough experimental re-evaluation [32,33] (see the corrected values in Table 1; entries 22, 41, 48, and 54). A typical mistake in estimation of kinetic parameters of enzymatic reactions is a neglecting the dilution

Table 1
Enzymatic properties and kinetic parameters for fungal β -galactosidases

Strain	MW (kDa) (units)	pI	pH opt. ($^{\circ}$ C)		T. opt. ($^{\circ}$ C)	K_M (mM) ^a		MCR Eq. (2)	Reference	
			NPG	Lactose		NPG (o/p)	Lactose			
β-Galactosidases from moulds										
1	<i>Alternaria tenuis</i>	142	4.6	3.8–4.4	3.6–4.8	60–65	0.21 (o)	6.57	31.3	[83]
2	<i>Alternaria alternata</i>	125		4–4.5	5–5.5	60	1.56 (o) ^b	13.9	8.9	[84]
3	<i>Aspergillus aculeatus</i>	120	5.3–5.7	5.4	5.4	55–60	3.28 (p) ^b	34.8 ^b	10.6	[44]
4	<i>Aspergillus foetidus</i>	126 \pm 2		2.2–4.5	3.5–4.0	66–70	1.613 (p)	68.8 ^c	42.7 ^c	[66]
5	<i>Aspergillus fonsecaeus</i>	126	4.2	2.6–4.5	2.6–3.0		1.78 (o)	61.03	34.3	[85]
6	<i>Aspergillus nidulans</i>	450 (97/120 \times 4)		7.5		30	1.4 (p) ^b	71 ^b	50.7	[24]
7	<i>Aspergillus niger</i>	124	4.64	2.5/3.5	3.0	60	2.35 (p)	85	36.2	[86]
8							27 (o) ^{b, d}			[71]
9							3.1–3.3 (p)			[87]
10			4.3	2.8/3.8		55	2.22 (p)			[73]
11		117	4.9				2.02 (o)			[88]
12				4			1.63 (o)			[89]
13		93		4.5		60–65	1.3 (p)			[90]
14		300 (130 \times 2)		4–4.5		65	1.2 (p)			[91]
15				3.2–4.6			1.00 (p)			[63,92]
16								81/99.5 ^{e, f}		[76]
17								86		[93]
18								53.9 \pm 6.6 ^e		[77]
19					4.5	50		22–33 ^{a, g, h, i}		[52]
20			4	4		55		20		[64]
21					3.5–4			6.2 ^b		[62]
22	<i>Aspergillus oryzae</i>		4.2	4.0–4.5		50–55	1.49–1.57 (o/p) ^{a, g, h}	52 ^{a, g, h}	33.1	[32,33,35,94,95]
23							1.1 (o) ^{a, e}	90 ^{a, e}		[96]
24				4.5			0.9 (o)	75.3		[27]
25		102		4.5–4.75	4.75	60	1.61 (o)	69.36		[97]
26		90	4.5	5.0	5.0	50–55	0.77 (o)	50		[98]
27		110	4.6	4.5–4.8	4.5	53	1.8 (o)/1.3 (p)	38.0		[99]
28		105		4.5	4.8	46	0.72 (o)	18		[100]
29				4.5			1.79 \pm 0.11 (o)			[101]
30				5			1.64 (o)			[89]
31							1.0/1.2 \pm 0.2 (p)			[48]
32							0.97 (o)			[102]
33								98 ^h , 132 ^a		[103]
34					4.5	50		52.78		[104]
35								49 \pm 5		[105]
36					4–4.5	55		46.9		[20]
37								46.9 ^g		[106]
38								42.75 ^e		[107]
39					4.5	50		42		[108]
40					4.6	48		21.4 ^g		[31,109]
41	<i>Curvularia inaequalis</i>		4.4	4	4.7	60	0.57 (o/p) ^{a, g, h}	20.6 ^{a, g, h}	36.1	[32,33,35,94,95]
42		115/126	4.4	3.7–4.5	3.9–5.3	60	0.55 (o)	4.5		[19]
43							0.56 (o)	7.3		[110]
44	<i>Fusarium moniliforme</i>			3.8–5.0	3.8–5.0	50–60	4.4 (o) ^b	12.4	2.8	[78]
45	<i>Fusarium oxysporum</i>	224 (36.3 \times 6)	3.83	5		55	6.76 \pm 0.26 (o) ^b			[111]
46	<i>Macrophomina phaseoli</i>	109–117	3.6	5	5	60	0.45 (o)	15	33.3	[112,113]
47	<i>Neurospora crassa</i>	96	4.82	4.2			0.38–0.62 (o)			[16,17,68,69,114]
48	<i>Penicillium canescens F178</i>		5	4.2	4.2	55	0.48 (o/p) ^{a, g, h}	15.9 ^{a, g, h}	33.1	[32,33,35,94,95]
49		120–126	6.7	4.5	4.5	55	0.5 (o)	14		[21]
50	<i>Penicillium chrysogenum</i>	270 (66 \times 4)	4.6	4		30	1.81 (o) ^b			[67]
51	<i>Penicillium citrinum</i>	100/110 (60 \times 2)	6.4	4.5	4.5	50	1.7 (o/p) ^b	25	14.7	[115]
52								20.4 ^g		[106]
53	<i>Penicillium frequentans</i>			4		62	2.06 (o) ^b			[116]
54	<i>P. multicolor (Lactase P)</i>					55	0.57 (p) ^{g, h}	20 ^{g, h}	35.1	[32,95]
55		126/130	5.9	4.0	4.0	60	0.6 (o)	8.9		[117]
56							0.46 (o)			[102]
57								10.5 ^g		[106]
58	<i>Penicillium</i> sp.						1 (p) ^b	11.1	11.1	[118]
59	<i>Pycnoporus cinnabarinus</i>	110		2.4	2.4		0.95 (p)	33	34.7	[119]
60	<i>Rhizomucor (Mucor) pusillus</i>	129/131	4.55	4		65	0.609 (o)/0.374(p)	22.5	36.9/60	[25]
61	<i>Rhizomucor</i> sp.	250 (120 \times 2)	4.2	4.5	4.5	60	1.32 (o)/0.66 (p) ⁱ	50 ⁱ	38/76	[28]
62	<i>Sclerotinia sclerotiorum</i>	120	6.0	4.0		50	0.5 (p)			[120]

Table 1 (Continued)

Strain	MW (kDa) (units)	pI	pH opt. (°C)		T. opt. (°C)	K_M (mM) ^a		MCR Eq. (2)	Reference
			NPG	Lactose		NPG (o/p)	Lactose		
63 <i>Sclerotium (Corticium) rolfsii</i>			2–2.5			0.384 (p)			[70]
64	221		2.7		60	3.7 (o) ^b			[65]
65 <i>Sclerotium tuliparum</i>	120–138	4.5	2	2	53	1.4 (o) ^b	20	14.3	[113,121]
66 <i>Thermomyces lanuginosus</i>	150–160/200–220 (75–80 × 2)	4.4–4.5	6.7–7.2			11.3 (o)/4.8 (p) ^{b,g}	18.2 ^{b,g}	1.6/3.8	[23]
67 <i>Trichoderma reesei</i>			4.6		63	0.4 (o)			[122]
β -Galactosidases from yeasts									
68 <i>Candida pseudotropicalis</i>			6.2	6.2	45–47.5	3.2–3.4 (o)	32.6–33.7	10	[123]
69 B57	200		7.5		30	3.45 (o)			[124]
70			6.2		45	3.06 (o)			[125]
71 A60	215		7.5		30	2.82 (o)	16.7		[124]
72 <i>Kluyveromyces (Saccharomyces) lactis</i>	200 (114 × 2)				35–40	1.7 (o)	17.3	10.2	[126]
73	135 × 2		7.25			1.61 ± 0.06 (o) ^e	16.8 ± 0.1 ^{e,k}	10.4	[43]
74			7.0	7.0	30–35	3.62 (o)	39.8	11.0	[127]
75			7.0	6.5		1.25 (o) ^a , 1.5 (o) ^g	29 ^a , 28 ^g		[128]
76			6.5–7.0	6.5–7.0	35	2.5 (o)	20.8		[129]
77	230 (118 × 2)		7	7	37–40	1.5 (o)	20/21 ^e		[130,131]
78				6.8			76.9 ^e		[132]
79				7.0	40		35–39 ^{a,g,h,i}		[52]
80							30		[133]
81							21 ^g		[106]
82				7.0			16		[134]
83				6.5	40		13.3 ^j		[135]
84							2.536 ± 0.412 ^{a,e,i}		[41]
85					55	3.03 (o) ^l			[49]
86	550		7			2.3–2.8 (o) ^m			[136]
87						2 (o) ^{a,h,i}			[137]
88				6.6–7.0	40	1.74 (o)			[138,139]
89			7.2			1.18 (o)			[140]
90 <i>Kluyveromyces (Saccharomyces) fragilis</i>			6.5	6.5	37	2.5 (o)	24	9.6	[18]
91			6.5–7.0	6.5–7.0	40	1.9 (o)	22.9		[129]
92	203	4.4	6.8			4.0 (o)	21.0		[141]
93	201		6.2–6.4			2.72 (o)	13.9		[142]
94			7	7	35	1.37 (o)	13.33		[143]
95							43.6 ^e		[40]
96				6.5	40		4.6–23 ^{e,n}		[42]
97							16 ^g		[106]
98							6–7 ^e		[14]
99	130		6.5–6.7			2.97 (o)			[39]
100						2 (o) ^{a,i}			[144]
101			6.5		50	1.7 (o)			[145]
102			7			1.34 (o)			[89]
103	>500		6.7–7.1		30–35	0.0056 (o/p)			[146]
104 <i>Kluyveromyces marxianus</i>	280		6.2		45–52	3.1 (o)	25	8.1	[51]
105			7.5	7.5	45–50	5 (o)	40 ⁱ		[50]
106							24.8–31.7 ^o		[13]
107				6.6			21–27.8 ^p		[26]
108 <i>Sporobolomyces singularis</i>	140–145		3.9–4		45–50	50 (o) ^{b,j}			[72]
109 <i>Tolulopsis sphaerica</i>	233		7		30	2.09 (o)	25.0	12.0	[124]
110 <i>Tolulopsis versatilis</i>	226		7		30	3.45 (o)			[124]
111 <i>Trichosporon cutaneum</i>	120	6.5–7	6.5–7	37	0.75 (o/p) ^b	20	26.7		[22]
	180 (2)/400 (4)								

^a Estimated by Lineweaver–Burk plot [11,12,15], if not indicated otherwise.

^b See Sections 2 and 3.

^c Recalculated as 61 mM using Hanes–Wolf and direct linear plots (see Section 2), which leads to MCR = 38.

^d Estimated by Bar-Eli–Katchalski method [71].

^e Estimated by non-linear regression methods.

^f At 30 °C for two kinetic models; for the same models K_M varied 83.3–78.2/101.9–96.9 over the interval 8–60 °C [76].

^g Estimated by Hanes–Wolf plot [11,53,54].

^h Estimated by Cornish–Bowden–Eisenthal (direct linear) plot [3,56,57].

ⁱ Estimated by Eadie–Hofstee plot [11,55].

^j The method of K_M estimation is unknown.

^k Found when glucose formation was measured; the value of 12.2 mM was obtained from galactose formation [43].

^l K_M was 1.25 mM, when cell growth media contained 15% lactose [49].

^m Estimated using Mbuyi–Kalala plot [136].

ⁿ At 25–40 °C; $K_M = 0.4$ mM at 5 °C [42].

^o At 25–30 °C; K_M varied from 15.4 to 38.3 over the interval 10–37.5 °C [13].

^p At 28–35 °C [26].

Table 2
Enzymatic activities and Michaelis constants for selected β -galactosidases

Vendor ^a	Activity (U/g prep.)			K_M (mM) ^b			V_{max} (U/mg protein) ^{bc}		
	<i>o</i> NPG	<i>p</i> NPG	Lactose	<i>o</i> NPG	<i>p</i> NPG	Lactose	<i>o</i> NPG	<i>p</i> NPG	Lactose
<i>Aspergillus oryzae</i> [32,33,35,95]									
A	400	240	75		1.49			1.1	
B		6000	1600		1.56				
C		22000	5000		1.57	52		95.2	50.3
<i>Curvularia inaequalis</i> [32,33,35,94]									
D	4200	3000	1660	0.58	0.56	19	25.0	17.3	13.2
D	5000	4000	1750	0.57	0.56	20	29.7	24.6	18.4
D	7400	5300	1800	0.55	0.57	22	38.4	28.1	20.7
<i>Penicillium canescens</i> [32,33,35,94,95]									
A	2000	1910	630		0.47	15		48.8	25.1
A		10700	2500		0.48	16			39.6
E	200000 ^d			0.5 ^d		14 ^d			

^a A—the All-Union Research Institute for Biotechnology; B—Sigma Co.; C—Röhm Co.; D—the All-Union Research and Technology Institute for Medical Antibiotics and Enzymes; E—the All-Union Research Institute for Genetics and Selection of Industrial Microorganisms.

^b Apparent K_M and V_{max} were obtained from Lineweaver–Burk [11,12,15], Hanes–Woolf [11,53,54], and Cornish–Bowden–Eisenthal [3,56,57] plots.

^c V_{max} are expressed in mmol of hydrolyzed substrate per min per mg of protein.

^d Ref. [21].

of a hydrolyzed sample before a measurement. This might be an explanation, for instance, of why $K_M(oNPG) = 4.5$ mM reported earlier for the β -galactosidase from *Neurospora crassa* [68] was later corrected to 0.45 mM [69] (though, a common misprint could be also a reason). Other obvious examples causing the same suspicion are the K_M values for enzymes from *Sclerotium (Corticium) rolfsii* (0.384 mM [70] and 3.7 mM [65] with NPG), and from *A. niger* with *o*NPG (27 mM [71], nearly 10 times overestimated), and with lactose (6.2 mM [62], nearly 10 times underestimated). The value $K_M(oNPG) = 0.75$ mM reported for the enzyme from *Trichosporon cutaneum* [22] is two to three times lower than for any other yeast β -galactosidase (Table 1), while $K_M(oNPG) = 50$ mM for the *Sporobolomyces singularis* enzyme [72] is approximately 20 times exaggerated. The values of K_M for several other enzymes with NPG as a substrate (Table 1, entries 3, 44, 45, 50, 51, 65, and 66) are unreasonably large when compared to the data for relative enzymes, especially the ones, which have similar K_M values with lactose. Since these $K_M(NPG)$ are respectively about 2, 10, 10, 2, 2, 2, and 10 times larger than should be expected, this may be an indication of an overlooked dilution factor.

Some other factors, e.g. transglycosylation [29,41,44], substrate inhibition [44,73], mutarotation of lactose [74], product inhibition by *o*-nitrophenol [29,75] or galactose [30,40,41,64,74,76], mutarotation of β -galactose to a stronger inhibitor α -galactose [74,76,77], may also contribute to inaccuracies of K_M estimations, if appropriate precautions have not been taken.

Although the range of reported K_M variations is very wide for some enzymes, these variations are not evenly distributed over the range. There is always a cluster of the parameter values close to a median, indicating an existence of a “true”, or “best” apparent K_M value for a particular enzyme–substrate combination.

Thus, we had to select the better data for further consideration, especially when numerous estimations had been published. Being interested in $K_M(\text{lactose})/K_M(\text{NPG})$ ratio, we focused on publications reporting K_M for both standard substrates. The data reported for only one substrate were considered as an additional material for substantiating our choice. A closeness of data obtained by different authors for the same enzyme and substrate(s) was an important argument in favor of these data. In case of diverging K_M values obtained by different linearization methods, we preferred the estimations made or supported by graphical methods other than Lineweaver–Burk plot (see above). The K_M values for enzymes from *Aspergillus aculeatus* [44], *Aspergillus nidulans* [24], *Fusarium moniliforme* [78], *Thermomyces lanuginosus* [23], and *Trichosporon cutaneum* [22] (entries 3, 6, 44, 66, and 111 in Table 1) have been excluded from further consideration as probably insufficiently accurate. In addition to the reasons discussed above, the following was taken into account for these enzymes. An instability of *o*NPG at the temperature used for the hydrolysis measurements (50 °C [78]) should cause a systematic error. The enzyme from *A. nidulans* was very unstable at temperatures above 10 °C and had unusual pH and temperature optima [24]. Unusual pH optimum was also reported for *T. lanuginosus* enzyme [23]. The values $K_M(\text{lactose})$ for *A. aculeatus* [44] and *T. lanuginosus* [23] enzymes were determined from the data on galactose formation, which are known [43] to produce lower K_M values than the ones based on glucose formation (an overwhelming majority of the available data). Besides, the authors mentioned [44] an interference of transglycosylation and substrate inhibition with the determination of enzyme activity.

The K_M values selected this way for further discussion are shown in bold (Table 1). Noteworthy, when a few published estimations are available, these “best” values are virtually the median ones.

Table 3
Amino acid composition of fungal β -galactosidases^a

	Typical ^b	<i>A. tenuis</i> [83]	<i>A. niger</i> [86]	<i>A. oryzae</i> [99]	<i>M. phaseoli</i> [113]	<i>N. crassa</i> [68,69]	<i>P. canescens</i> [21]	<i>P. citrinum</i> [115]	<i>P. multicolor</i> [117]	<i>Mucor</i> (<i>Rhizomucor</i>) <i>pusillus</i> [25]	<i>Rhizomucor</i> sp. [28]	<i>S. tulip.</i> [113]	<i>K. fragilis</i> [153]	<i>S. singularis</i> [72]	<i>E. coli</i> [154]
Ala	6.7–9	<i>11.0</i>	6.8	6.7	7.8	9.0	8.5	7.1	7.2	9.9	<i>10.7</i>	7.4	5.2	<i>10.5</i>	7.9
Arg	2.6–3.5	3.0	3.2	3.4	2.6	<i>0.0</i>	3.0	2.7	2.2	<i>1.8</i>	2.2	3.0	3.3	3.5	<i>6.3</i>
Asp	10.6–11.6	7.5	10.9	11.2	10.6	8.2	11.2	11.1	11.4	11.5	<i>13.3</i>	11.1	<i>12.5</i>	11.6	10.5
Cys	0.3–0.5	–	0.3	0.4	<i>0.1</i>	–	0.4	0.4	0.4	0	0.3	0.5	2.3	0	<i>1.6</i>
Glu	7–10.8	10.6	9.2	8.7	8.4	<i>13.7</i>	8.5	8.1	8.3	7.5	7.7	7.9	10.8	10.2	<i>12.1</i>
Gly	9.4–11.1	10.5	9.4	10.2	10.4	25.2	11.1	10.0	10.8	<i>16.8</i>	10.5	10.8	6.9	10.9	7.2
His	1.4–2.1	2.1	1.6	1.6	2.1	1.4	2.9	1.9	1.9	1.6	<i>0.9</i>	1.4	3.8	2.4	<i>3.1</i>
Ile	3.9–5	3.9	4.3	4.1	4.7	<i>1.4</i>	<i>3.4</i>	4.6	<i>3.4</i>	5.0	4.6	5.6	5.4	4.8	4.1
Leu	6.4–8.8	7.7	9.6	8.8	8.4	2.5	7.8	7.9	8.6	6.6	6.4	7.0	7.9	8.0	<i>9.4</i>
Lys	4.3–5	4.7	3.6	4.3	3.5	7.9	4.6	5.8	5.0	4.6	4.6	3.4	7.6	5.8	2.5
Met	0.6–0.9	0.9	0.9	0.9	<i>0.4</i>	<i>0.1</i>	<i>0.3</i>	0.6	0.6	0.6	<i>1.4</i>	0.7	0.6	0	<i>2.0</i>
Phe	3.6–5.8	8.5	4.5	4.7	4.4	<i>0.7</i>	4.2	4.6	3.8	3.6	5.7	4.5	5.8	5.3	3.8
Pro	4.7–7.1	8.8	5.8	6.6	6.1	2.5	5.1	7.1	5.4	5.5	4.7	5.0	4.7	5.8	5.7
Ser	6.7–10.3	9.0	7.5	7.4	7.5	<i>19.7</i>	10.3	8.5	9.5	7.8	6.7	9.1	4.9	7.0	5.7
Thr	6–7.5	5.2	7.5	6.4	8.2	4.2	6.9	6.0	6.9	7.2	6.5	8.0	4.6	6.7	5.5
Trp	1.7–2.7	–	2.0	2.3	2.7	–	<i>0.5</i>	1.7	2.5	–	2.4	2.0	2.0	–	<i>3.0</i>
Tyr	4.7–6.1	<i>1.2</i>	6.1	6.7	5.1	<i>1.3</i>	5.4	5.6	6.0	3.6	4.7	5.4	5.1	1.8	<i>3.1</i>
Val	5.3–6.9	5.4	6.8	5.6	6.9	2.2	5.3	6.5	6.1	6.5	6.8	7.2	6.4	5.8	6.4
Residues per mol		764		894	886	1029		521 × 2	838			912	1412	1072	1173

^a Expressed in mol%.

^b A cluster of values encompassing the data for not less than a half of the listed enzymes (Fig. 1); deviations from a “norm” are shown in italics.

The dependence of K_M values on the structure of substrate (aglycon) is obvious when the data for lactose and NPG are compared. However, K_M is practically the same for the hydrolysis of *o*NPG and *p*NPG by fungal β -galactosidases (Tables 1 and 2) (enzymes from *Rhizomucor* genus may be an exception). Equal values of K_M were found for *o*NPG and *p*NPG also in case of the non-fungal β -galactosidases from *Tritrichomonas foetus* [79], *Rhizobium meliloti* [80], *Thermoanaerobacterium thermosulfurigenes* [81], and from bovine testes [82]. Therefore, we used equally K_M for *o*NPG and *p*NPG (Table 1) for further discussion. An attempt to consider these data separately has merely lead to two generally equivalent sets of parameters.

3. Discussion

3.1. Enzyme purity

Hypothetically, “the purification of enzyme preparations could result in a decrease in K_M value if any possible inhibitor present in these preparations is removed” [132]. However, the available data for fungal β -galactosidases (Table 2) attest to the independence of K_M on enzyme purity/activity, since the same values of K_M have been measured by three major graphical methods for several samples of each fungal β -galactosidase from different vendors and/or of different purity. For instance, even a 100-fold difference in enzyme activity does not change K_M in case of *P. canescens* and *A. oryzae*. Noteworthy, K_M does not depend on the enzyme purity also in case of covalently immobilized β -galactosidases [94].

For the same samples of enzymes, the V_{max} values depend on both their purity and on substrate structure. However, we found that the ratio $V_{max}(oNPG)/V_{max}(pNPG)$, as well as the ratio of corresponding activities, was approximately 1.2–1.4 for the β -galactosidases from *C. inaequalis* (Table 2), *Macrophomina phaseoli* [112], *Penicillium citrinum* [115], and *T. cutaneum* [22]. For the β -galactosidases from *P. canescens* (Table 2) and *P. multicolor* [117], the ratio of activities was 1.1. This regularity can be used for a rough estimation of $V_{max}(oNPG)$ or $V_{max}(pNPG)$, if only one of these parameters has been measured. The ratio $V_{max}(NPG)/V_{max}(\text{lactose})$ was also approximately constant for different samples of *C. inaequalis* (Table 2), but it was different for other β -galactosidases mentioned above.

3.2. Enzymatic properties

A comparison of enzymatic properties for the mould β -galactosidases (Table 1) reveals several important similarities: close molecular masses (typically around 120 kDa), pH optima ($\sim 3.5 \pm 1.5$), temperature optima for natural and synthetic substrates (~ 50 – 60°C), and isoelectric points [$\sim 4.5 \pm 0.5$; except for *A. aculeatus* (5.3–5.7), *M. phaseoli* (3.6), *Sclerotinia sclerotiorum* (6.0), and three enzymes of *Peni-*

cillium group (5–6.7)]. The group of yeast β -galactosidases differs from the mould enzymes in molecular masses (typically around 200 kDa), pH optima (~ 6.5 – 7), and temperature optima (~ 35 – 50°C).

3.3. Amino acid composition

When searching for similarities of enzymes, it is instructive to compare their amino acid sequences. Unfortunately, there are only three mould (*A. niger*, *P. canescens* F-178, and *Penicillium* sp. in GH35 family) and two yeast (*K. lactis* and *K. marxianus* in GH2 family) β -galactosidases with known K_M values present in Henrissat’s classification of glycosyl hydrolases based on amino acid sequence similarities [147–151] (<http://afmb.cnrs-mrs.fr/CAZY/>; updated on 23 June 2004). Therefore, we compared the published data on amino acid composition of fungal β -galactosidases (Table 3), and found a substantial similarity. Such a likeness was noticed previously for three β -galactosidases of genus *Penicillium* [21], and for enzymes from *Mucor* (*Rhizomucor*) *pusillus* and *A. niger* [25]. Similarity of amino acid composition was also found within a series of bacterial β -galactosidases [152]. Using the data in Table 3, we were able to find a typical range for each amino acid—a central cluster of close values encompassing not less, usually more than a half of the fungal enzymes listed (Fig. 1).

The most significant deviation of amino acid molar percentage from the typical values is observed for *N. crassa* (for at least 14 of 18 amino acids). Essential deviation is observed for *K. fragilis* enzyme (9 of 18). For all other fungal enzymes, not more than one third of amino acids deviates from the typical values. The amino acid molar content for *A. niger*, *A. oryzae*, *P. citrinum* and *P. multicolor* is almost perfectly typical. Two yeast enzymes, from *K. fragilis* and *S.*

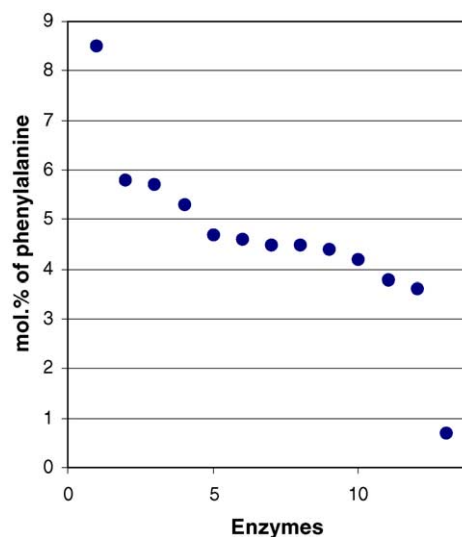


Fig. 1. Estimation of a typical range of amino acid percentage for fungal β -galactosidases exemplified by the data for phenylalanine (3.6–5.8 mol%). The order of enzymes does not correspond to the one in Table 3.

singularis, do not look alien to the mould majority of fungal β -galactosidases. Noteworthy, the typical composition of fungal β -galactosidases is significantly different from the one for bacterial enzyme from *E. coli* (for 11 of 18 amino acids).

3.4. Michaelis constants ratio—relative substrate affinity

Examination of Table 1 reveals two broad groups of fungal β -galactosidases: the enzymes with large K_M values and the ones with small K_M values. *Aspergilli* constitute a core of the first group, and *Penicillium* genus is a core of the second group. *Rhizomucor* sp. adjoins the *Aspergillus* group. All other β -galactosidases, including yeast enzymes, are closer to *Penicillium* group. The relatively low K_M (the higher rate of reaction) makes the last group of β -galactosidases more useful for practical applications, e.g. for hydrolysis of lactose in whey. This difference is especially important for immobilized β -galactosidases, since immobilization often increases K_M significantly [9,26,40,52,94,96,108,137–139,145,155].

Using the collected literature values for K_M , we estimated the Michaelis constants ratio (MCR, Eq. (1)) for a series of fungal β -galactosidases with two substrates: a natural substrate—lactose, and a synthetic (model) substrate—*o*- or *p*-nitrophenyl- β -D-galactopyranoside (*o/p*NPG) (Table 1). The selection of reliable data for this estimation is described in Section 2 and partially below. Although K_M values can be substantially different for the mould β -galactosidases from different strains, the parameter $MCR_{(lactose/NPG)}$ (Eq. (2)) is approximately constant for this group of enzymes, and its average value equals 35 ± 3 for 12 enzymes selected from 20 strains, for which both K_M values are known (Table 1). Similarly, we found $MCR_{(lactose/NPG)} = 10 \pm 1.5$ for a set of five yeast β -galactosidases selected from six with both K_M values known (Table 1). Importantly, the MCR values for the mould enzymes and for the yeast enzymes are essentially different despite an overlap of their K_M ranges.

$$MCR_{(lactose/NPG)} = \frac{K_M(lactose)}{K_M(NPG)} \quad (2)$$

Another estimation of MCR was obtained from a linear correlation (through origin) $K_M(lactose)$ versus $K_M(NPG)$ (Figs. 2 and 3). The values obtained are 36.5 for the mould enzymes, and 9.7 for the yeast enzymes, which is well within the limits of the estimated standard deviation for the average values described above. The difference between mould and yeast β -galactosidases becomes especially obvious when the data are visualized (Fig. 3).

Four outlying points are shown at Fig. 2 for the mould β -galactosidases from *Alternaria alternata*, *P. citrinum*, *Penicillium* sp., and *Sclerotium tuliparum* (Table 1, entries 2, 51, 58, and 65). The published information on the conditions of measurement and calculation was scarce and did not give a reason for canceling of these enzymes at the step of preliminary data evaluation (see Section 2). Nonetheless, they were excluded when the diagram was drawn, since these points split far away from the excellent correlation for the major

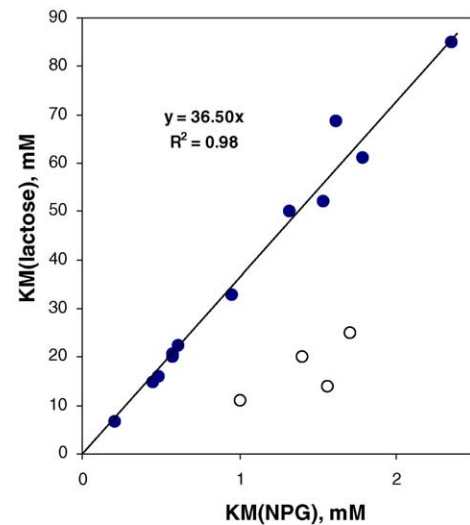


Fig. 2. Correlation of $K_M(lactose)$ vs. $K_M(NPG)$ for the mould β -galactosidases [Table 1, entries ● (from left to right): 1, 46, 48, 41 + 54, 60, 59, 61, 22, 4, 5, 7; entries ○ (from left to right): 58, 65, 2, 51].

group of the mould enzymes. Hypothetically, these enzymes could belong to a separate group of β -galactosidases with a different MCR. However, it is hard to combine in one special group the enzymes from so different genera. We believe that these deviations are the results of experimental or calculative errors of the types discussed in Section 2. Apparently, the $K_M(NPG)$ values have been somehow overestimated.

Thus, the MCR parameter offers a new instrument for the identification of certain types of enzymes and their classification. In general terms, MCR for a group of enzymes with a pair of standard substrates (e.g. a natural and a model ones) may serve as an identification number or a “trademark” of this group. In addition to different MCR for the mould and yeast β -galactosidases, we found preliminarily two groups

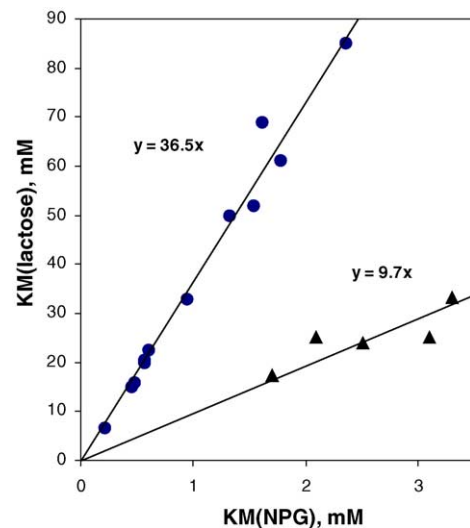


Fig. 3. Comparison of correlations $K_M(lactose)$ vs. $K_M(NPG)$ for the mould (●) and yeast (▲) β -galactosidases.

of the bacterial β -galactosidases with $MCR_{(\text{lactose}/\text{NPG})}$ in the intervals 2–4 and 8–12, and two groups of the plant enzymes with $MCR_{(\text{lactose}/\text{NPG})}$ within 10–20 and 60–80. For the mammalian β -galactosidases $MCR_{(\text{lactose}/\text{NPG})}$ is close to or exceeds 200. This comparison makes two groups of fungal enzymes (mould and yeast) look even more coherent.

Since we are suggesting a new parameter for identification of related enzymes, it is instructive to discuss a (potential) correspondence between the MCR-based groups of enzymes and the families in the extremely important Henrissat classification of glycosidases by amino acid sequence comparisons [147–151] (<http://afmb.cnrs-mrs.fr/CAZY/>; updated on 23 June 2004). There are currently only six examples of mould β -galactosidases in this database (*Aspergillus candidus* 88, *A. niger*, *Hypocrea jecorina*, *P. canescens* F-178, *Penicillium* sp., and *Talaromyces emersonii*—all in GH35 family), and two yeast enzymes (*K. lactis* and *K. marxianus*—both in GH2 family). The β -galactosidase from *A. aculeatus* was tentatively assigned to GH35 family [44], because its 18 N-terminal amino acid sequence matched with *A. niger* enzyme. According to the results of present study, the mould *A. niger* and *P. canescens* β -galactosidases belong to the group with $MCR_{(\text{lactose}/\text{NPG})} \approx 35$ (we believe, the coincidence with the family GH35 number is purely accidental). The yeast β -galactosidases from *K. lactis* and *K. marxianus* belong to the group with $MCR_{(\text{lactose}/\text{NPG})} \approx 10$. It seems reasonable to expect that all other mould β -galactosidases with $MCR_{(\text{lactose}/\text{NPG})} \approx 35$ should be in GH35 family, and all yeast β -galactosidases with $MCR_{(\text{lactose}/\text{NPG})} \approx 10$ should be in GH2 family, but we have yet to wait for more proofs. If more matches are found, MCR could be used as an auxiliary identification parameter.

We would like to emphasize that MCR is not a theoretical concept, but a regularity (proportion) found for the experimentally determined K_M parameters. However, a physical meaning of MCR is worth a brief discussion. The apparent Michaelis constant is often routinely considered as an “affinity parameter” (in fact, reciprocal to the affinity) for a semi-quantitative comparison of enzyme affinity to various substrates [1,4,5,11–31], and even as an equivalent to dissociation constant [1,4,5,12–15]. This meaning follows from the steady-state derivation of K_M in classical Michaelis–Menten kinetics as the sum of the constants for breakdown of enzyme–substrate complex, k_{-1} and k_2 , divided by the constant for its formation, k_1 (Eq (3)).



Even though K_M is approximately equal to the dissociation constant of the enzyme–substrate complex only in some cases when k_{-1} is much greater than k_2 , it can be generally considered as a reasonable characteristic of enzyme–substrate affinity. Then, it would be appropriate to interpret the Michaelis constants ratio for the same enzyme with two substrates as a characteristic of a “relative substrate affinity”.

The physical meaning of the Michaelis constant is more blurred if the reaction mechanism is more complex than that shown in Eq. (3). The observed K_M becomes a complicated algebraic combination of many rate constants [10]. In depth studies of enzymatic mechanisms are performed by fitting of experimental data to complex kinetic models by non-linear computer regression [61] (for some recent examples see [14,26,29,30,40–42,75,76,156]). Not surprisingly, this approach produces sometimes substantially different K_M values for different kinetic models [30,40,42,76,156]. Thus, the $K_M(\text{lactose})$ values of 76.9, 16.8, and 2.5 mM have been reported for *K. lactis* enzyme (Table 1). However, a comparative study of various kinetic models has shown that “for simplicity the conventional Michaelis–Menten equation with competitive product inhibition may be used to model the lactose hydrolysis reaction” catalyzed by the mould β -galactosidase from *A. niger* [76]. The same model with galactose inhibition was advocated by the results of kinetic studies for the yeast β -galactosidases from *K. fragilis* [14,40,42] and *K. lactis* [41]. Similar results were obtained recently for *Thermus* sp. β -galactosidase [30]. Since the standard methods for the apparent K_M determination imply the measurements at the initial period of reaction, the product inhibition by galactose (or nitrophenol) can be reasonably neglected. This means that the selected kinetic model mentioned above is effectively reduced to the simplest model expressed by Eq. (3).

Using the interpretation of MCR as a “relative substrate affinity”, one can say that the affinity of various mould β -galactosidases to NPG is approximately 35–36 times greater than their affinity to lactose, and for the yeast β -galactosidases this factor is about 10. Very importantly, the proportion remains nearly the same for different mould β -galactosidases, though the affinity to a particular substrate may vary essentially from enzyme to enzyme. The same regularity is less striking for the yeast enzymes due to a much narrower range of K_M .

This observation can be roughly interpreted as follows. The binding sites of various mould β -galactosidases may have the same or similar topology (a similar sequence and/or structural arrangement of participating amino acid residues), but different conformations (a degree of preparedness for binding). This difference in conformations may be responsible for the difference between enzymes in a magnitude of their affinity to the same substrate (hence, in the values of K_M), since some enzymes need more extensive conformational changes to bind a substrate by induced fit, than others [2]. At the same time, a similar set of binding functional groups may account for a similar preference towards one of two substrates, and hence for the constant relative substrate affinity (MCR). This interpretation is supported by the similarities in isoelectric points, pH- and temperature optima, and amino acid composition for this group of enzymes (see above). The same consideration should be valid for the yeast enzymes, but with different magnitudes of all parameters.

It is quite counter-intuitive that the affinity to an artificial substrate (NPG) is much greater than to a natural one

(lactose). There might be a special binding pocket for *o/p*-nitrophenoxy group in the structure of all β -galactosidases.

A closeness of K_M values (or V_{max} , k_{cat} , etc.) is often used to suggest a similarity of active centers for various enzymes [11,21,28,80,81,85,86,99,117,121]. The MCR parameter provides for the first time an indication of possible intrinsic similarity for enzyme binding sites when the values of K_M are substantially different.

The evaluated MCR allows a rough estimation of K_M value for a second substrate, when this parameter is known for one of the two. This approach can be useful for an estimation of K_M for β -galactosidase with lactose, since the measurement of K_M with NPG is typically much easier and faster (although not more accurate). For example, using the published values K_M (NPG) (Table 1) we calculated the unknown values K_M (lactose) for β -galactosidases from *N. crassa* (~16 mM), *S. rolfisii* (~13 mM), *S. sclerotiorum* (~18 mM), and *Trichoderma reesei* (~14 mM).

Another useful application of MCR can be a check for accuracy of K_M values, when they are measured for both substrates. A substantial deviation of MCR from a typical value for a group of related enzymes can reveal a possibly erroneous estimation of K_M . Alternatively, it may be an indication of a wrong assignment of the enzyme to this group.

Active search for MCR for various enzymes and substrates is currently in progress.

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