ELSEVIER

Carbohydrate Research 282 (1996) 171–180

CARBOHYDRATE RESEARCH

Elucidation of the subsite structure of bacterial saccharifying alpha-amylase and its mode of degradation of maltose

Toshihiko Suganuma [a,*], Masatake Ohnishi [b], Keitaro Hiromi [c], Tomonori Nagahama [a]

[a] *Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, Korimoto, Kagoshima 890, Japan*

[b] *Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto Prefectural University, Sakyo-ku, Kyoto 606, Japan*

[c] *Department of Food Science and Technology, Faculty of Engineering, Fukuyama University, Fukuyama 729-02, Japan*

Received 16 May 1995; accepted in revised form 18 October 1995

Abstract

The subsite structure of bacterial saccharifying alpha-amylase (BSAm) was elucidated by two methods using a series of maltooligosaccharides labeled with [^{14}C]D-glucose at the reducing end. The rate parameter k_0/K_m and the cleavage frequency were obtained using the labeled substrates at sufficiently low concentrations to eliminate transglycosylation and condensation. This evaluation showed that the active center is composed of five subsites, with the catalytic site located between the 3rd and the 4th subsites from the nonreducing end. The evaluated affinity values of a subsite varied with the set of data used, which suggests some stimulation factor resulting from the chain length effect. The appearance of a time lag during the digestion of the poor substrate, maltose, was studied using radioactively labeled maltose (81.6 mM). Radioactive oligosaccharides larger than maltose were found at a significant level of more than 2% of the initial substrate in the digests, including a product peculiar to condensation, G-G*-G, as 8–10% of the maltotriose in the digests. This indicates that transglycosylation is a main side reaction (ca. 90%). A degradation pathway for maltose via maltosyl transfer was proposed, in which G_3 behaves as a kind of catalyst.

Keywords: Subsite structure; Alpha-amylase; Maltooligosaccharides; Transglycosylation; Maltosyl transfer

* Corresponding author.

SSDI 0008-6215(95)00365-7

1. Introduction

Alpha-amylases from various sources have some common sequences that contribute to the formation of the active center [1,2]. The three-dimensional structures of active centers comprising several tandem subsites and possible catalytic residues have been elucidated by X-ray analysis at high resolution [3–10].

Differences in the action patterns of endo-enzymes are attributable to differences in their subsite structures: the number of subsites, the location of the catalytic site, and the affinities of each subsite [11–20].

The primary sequence of saccharifying alpha-amylase from *B. subtilis* [EC 3.2.1.1] (BSAm) has been deduced from its nucleotide sequence [21]. Unlike the liquefying type, the enzyme possesses both glycosyl transfer and hydrolytic activity, and thereby shows an uncommon behavior for a monomeric enzyme: a lag of time course [22] and a sigmoidal dependence of the reaction rate on substrate concentration [23] with the poor substrate maltose. Therefore, an attempt to evaluate subsite affinities of this enzyme has failed [24].

Fujimori et al. have discussed, based on a computer analysis, that this sigmoidal curve must be due to the glycosyl transfer or the condensation activity of the enzyme [22], but it is unknown which of these types of reactions is actually operative.

In the present study, we aimed to evaluate subsite affinities of the enzyme by two methods, as well as to determine the contribution of transglycosylation and condensation activities to maltose degradation. For these purposes, quantitative analysis of products using reducing end-labeled maltooligosaccharides as substrates is a powerful approach. The unique action pattern of this amylase was analyzed based on its subsite structure.

2. Experimental

Materials.—The crystallized sample of saccharifying alpha-amylase from *B. subtilis*, prepared according to Fukumoto et al. [25], was a gift from Dr. Fujimori at Heian Women's College. The sample was free from contamination by other amylolytic enzymes. The concentration of the enzyme was determined spectrophotometrically, using the value of the absorption coefficient (1%, 280 nm) of 19.8 cm^{-1} [26] and the molecular weight of 41,900 [27]. Maltooligosaccharides, labeled with [^{14}C]D-glucose at the reducing end (G_2^* to G_6^*), were synthesized from [^{14}C]D-glucose and cyclomaltohexaose by the coupling reaction of *B. macerans* transglycosylase [28] and purified by paper chromatography [29]. Unlabeled maltose was purified by paper chromatography from the commercially obtained maltose (Hayashibara Biochemical Laboratories, Inc., Okayama), in order to eliminate any contamination of other saccharides.

Assay.— The enzyme reactions with labeled substrates were performed in a microtube in 0.02 M acetate buffer, pH 5.3, at 25 °C throughout this study: A portion (25 μL) of the reaction mixture containing the labeled substrate (ca. 15–77 μM, 10^4–10^5 cpm) and the diluted enzyme was taken at appropriate intervals, and was terminated with 25 μL of 0.05 M NaOH. The reaction products (ca. 30% extent of reaction) were separated

Table 1
Cleavage frequencies of maltooligosaccharides and the apparent first-order rate constants k_0/K_m in the reaction of *B. subtilis* saccharifying alpha-amylase (BSAm)

Cleavage frequency							e_0 (M)	s_0 (μM)	k_0/K_m (M^{-1} min^{-1})
	G–	G*							
i) [a]	1.00						4.58×10^{-6}	63	1.29×10
ii) [b]	1.00								
iii) [c]	1.00								
	G–	G–	G*						
i)	0.224	0.776					1.53×10^{-7}	77	1.12×10^{5}
ii)	0.223	0.777							
iii)	0.226	0.774							
	G–	G–	G–	G*					
i)	0.000	0.442	0.558				1.51×10^{-9}	50	1.03×10^{7}
ii)	0.025	0.418	0.557						
iii)	0.002	0.444	0.554						
	G–	G–	G–	G–	G*				
i)	0.000	0.116	0.729	0.155			5.04×10^{-10}	41	2.51×10^{7}
ii)	0.007	0.115	0.725	0.153					
iii)	0.000	0.015	0.967	0.018					
	G–	G–	G–	G–	G–	G*			
i)	0.000	0.000	0.554	0.446	0.000		3.78×10^{-10}	15	4.32×10^{7}
ii)	0.004	0.067	0.420	0.420	0.089				
iii)	0.000	0.007	0.492	0.492	0.009				

[a] i): Experimental values.
[b] ii): Calculated values by procedure (b) in Table 2.
[c] iii): Calculated values by procedure (c) in Table 2.

by multiple ascending paper chromatography (Whatman 3 MM) with a solvent composed of 65% *n*-propanol aq solution. After irrigation twice, the strip of paper (2 × 40 cm) was cut into segments (0.5 or 1.0 cm), which were placed in glass vials for scintillation counting [15]. All labeled products were identified by paper chromatography using authentic unlabeled maltooligosaccharides.

The rate of decrease in the labeled substrate can be assumed to obey first-order kinetics, since the reaction was performed at much lower substrate concentrations than K_m. The equation is

$$kt = \ln(s_0/s) \quad (1)$$

Thus, for example for the G_5^* reaction, the plot of $\ln(\Sigma G_i^*/G_5^*)$ vs. time is supposed to be linear, where ΣG_i^* represents the sum of the radioactivity of all radioactive species (G_1^* to G_5^*), i.e., the radioactivity of the initial amount of substrate G_5^* (s_0). From the slope, we can obtain the value of V/K_m as a first-order rate constant k, and that of k_0/K_m, by dividing it by the enzyme concentration e_0 [15]. The concentrations of enzyme and substrates employed are summarized in Table 1.

The reaction with a high maltose concentration was performed essentially as above, except for the addition of unlabeled maltose.

3. Results and discussion

Cleavage frequency and kinetic parameters (k_0/K_m).—There are some features noticeable for the mechanism of action of alpha-amylase other than hydrolysis, such as transglycosylation and condensation. These additional reactions complicate the interpretation of kinetic parameters and cause changes in the apparent cleavage pattern of oligosaccharides. In evaluating subsite affinities, it is necessary that enzyme assays are performed at sufficiently low concentrations of substrate, since at low concentrations the side reactions can be eliminated [15]. For the purpose of evaluating the significance of the above phenomena, we used a series of radioactive reducing end-labeled maltooligosaccharides (G_2 to G_6) as substrates, and determined the rate parameters k_0/K_m and cleavage frequencies. The frequency was determined from the relative radioactivity of the product corresponding to the bond-cleavage (Table 1).

Maltotriose was cleaved predominantly at the first glycosidic bond from the reducing end. Maltotetraose was also cleaved at the same glycosidic bond most easily. With higher saccharides than G_4, the internal bonds are most susceptible and bonds next to the ends are not readily hydrolyzed.

With labeled substrates at low concentrations, all plots of $\ln(s_0/s)$ vs. time gave straight lines, which implied that the reactions obeyed first-order kinetics (data not shown). The values of k_0/K_m were obtained from the plots. The value for maltose was much smaller, about 10^{-4} times than that of G_3, which in turn is about 10^{-2} times smaller than that of G_4. With higher saccharides, the hydrolytic rates did not differ so much. The cleavage property for G_4 and the rate dependency on the degree of polymerization (dp) were responsible for the characteristic behavior of this enzyme towards maltose as is discussed below.

Evaluation of subsite affinities.—The subsite structure of BSAm was elucidated by two evaluation methods. First, a revised method for evaluating subsite affinities [15] was applied. When the catalytic site is located between the rth and the $(r+1)$th subsites, a subsite affinity can be evaluated from the values of product frequencies and the kinetic parameters (k_0/K_m) according to the following equation:

$$\begin{aligned}\frac{k_n(i)}{k_{n-1}(i-1)} &= \frac{(k_0/K_m)_n\left([P_i^*]/\sum_{}^{n-1}[P_i^*]\right)}{(k_0/K_m)_{n-1}\left([P_{i-1}^*]/\sum_{}^{n-2}[P_i^*]\right)} \\ &= \frac{k_{int}K_{n,r+i}}{k_{int}K_{n-1,r+i-1}} \\ &= \exp(A_{r+i}/RT)\end{aligned} \tag{2}$$

where R and T are the gas constant and the absolute temperature, respectively. This equation means that the affinity of the subsite (A_{r+i}) occupied only by a longer substrate G_n^* could be obtained by the difference between the apparent rate constant $(k_n(i))$ of formation of P_i^* (or G_i^*) from G_n^* and that $(k_{n-1}(i-1))$ of P_{i-1}^* (or G_{i-1}^*) from G_{n-1}^*. The rate constant $(k_n(i))$ is proportional to the association constant $(K_{n,r+i})$

Table 2
Subsite affinities (kcal/mol) of BSAm by various evaluation procedures

A_1	A_2	$A_3 + A_4$	A_5	Procedure
0.82			0.68	(G_5)–(G_4) [a]
1.09	1.66	(3.63) [e]	0.92	(G_5), (G_3) [b]
2.48	3.08	(−0.61) [e]	2.35	(G_4)–(G_3) [c]
	5.09		4.35	(G_3)–(G_2) [d]

[a] Calculated from a combination of data, k_0 / K_m and cleavage frequency, for G_5 and G_4.
[b] From cleavage frequencies for G_5 and G_3 (Thoma's method).
[c] From a combination of data for G_4 and G_3.
[d] From a combination of data for G_3 and G_2. See Fig. 1.
[e] Each affinity of the two subsites adjacent to the catalytic site cannot be evaluated by these procedures alone, but the sum can be calculated from the value of K_{int}, 4100 M^{-1}, obtained by Shibaoka et al. [24].

for a corresponding productive complex from which the labeled i-mer product (P_i^*) is produced. By comparing the hydrolytic rates for other pairs of substrates differing in chain length by one glucose unit, we can obtain all the subsite affinities except for the two subsites adjacent to the catalytic site [15].

The results are summarized in Table 2. The number of subsites was estimated to be five for this enzyme. The catalytic site of the enzyme is located between the 3rd and the 4th subsites. The value of the affinity of a subsite was found to vary with the set of data from different substrates. In Fig. 1, three different sets used for evaluating the first subsite affinity, A_1, are illustrated by (a) a combination of binding modes of G_4 and G_5 to produce maltose, (b) a combination of binding modes of G_5 to produce maltose and G_3, and (c) a combination of binding modes of G_4 and G_3 to produce glucose. The combinations (a) and (c) are applications of the above method, and the combination (b) was that of Thoma's method [11]. From combinations (a), (b) and (c), A_1 values were

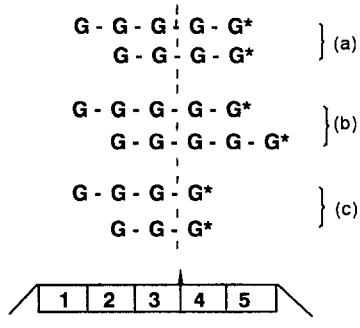

Fig. 1. A schematic representation for evaluation of a subsite affinity, A_1, in three different ways. The catalytic site of the enzyme is shown as a wedge. G and G* indicate unlabeled and labeled glucose residues, respectively. For details, see text.

determined to be 0.82, 1.09 and 2.48 kcal/mol, respectively. For example, in the case of (a),

$$RT \ln \frac{k_5(2)}{k_4(2)} = RT \ln \frac{(k_0/K_m)_5 \times \{[G_2^*]/([G_1^*] + [G_2^*] + [G_3^*] + [G_4^*])\}}{(k_0/K_m)_4 \times \{[G_2^*]/([G_1^*] + [G_2^*] + [G_3^*])\}}$$

$$= RT \ln[(2.51 \times 10^7 \times 0.729)/(1.03 \times 10^7 \times 0.442)]$$

$$= 0.82 \,(\text{kcal/mol}) \quad (3)$$

As seen in Fig. 1, the difference in procedures (a) and (c) resides in the occupation of the 5th subsite with a substrate molecule.

It is an intriguing question how the occupation of the 5th subsite causes the difference (2.48 − 0.82 = 1.66 kcal/mol) in the evaluated value of A_1. The occupation of the 5th subsite could possibly accelerate the chemical process for hydrolysis (lowering the activation energy for the catalytic process) or the binding process for other subsites (cooperativity between subsites). In the evaluation of A_5, the occupation of the first subsite could cause almost the same difference magnitude (2.35 − 0.68 = 1.67 kcal/mol, Table 2). Thus, the above difference would arise from the chain length effect [11] rather than the occupation effect of a specific subsite [30]. As for the other subsite affinity, A_2, the situation was similar. The value was dependent on the combination of the data chosen for the evaluation (Table 2).

Thoma et al. mentioned that the hydrolytic rate constant k_{cat} (or k_{int}) is dependent on the dp of a substrate with bacterial liquefying alpha-amylase [11]. They obtained the effect of occupation (chain length) as a correcting factor from data fitting, and estimated the average value of the lowering free energy to be 1.88 kJ/mol (0.45 kcal/mol) per subsite occupied.

Seigner et al. also reported similar results with porcine pancreatic alpha-amylase to the present study on BSAm: values of the affinity of a subsite obtained by different sets of substrates varied with the chain length beyond experimental errors [18]. Thus, the result is in conflict with the hypothesis of Hiromi et al., implying the independence of k_{int} on chain length and binding mode [13]. A recent study on porcine pancreatic alpha-amylase also reported that the binding of a glucose residue of substrate to subsite 5 directly affects the catalytic function of the enzyme to modulate kinetic parameters depending on pH [30].

In the case of BSAm, Thoma's evaluation method gave fairly good agreement between the experimental and the calculated values of cleavage frequency, especially for G_5^* (Table 1). As to our evaluation method, the calculated frequencies obtained from the values of A_i (procedure c in Table 2) was also in a good agreement with the experimental ones.

About the number of subsites.—Shibaoka et al. have estimated the number of subsites of the enzyme to be four, from the K_m values for longer substrates than G_3 [24]. The result of four subsites, however, cannot explain the cleavage patterns of G_4 and higher saccharides. For instance, G_4 is cleaved mainly at the two glycosidic bonds, the first and the second from the reducing end, and G_5 has a single preferential point of cleavage by the enzyme (Table 1). This implies that five subsites of the enzyme are required.

Why has such a difference in the number of subsites arisen between the two studies? The difference is considered to arise from their application of the following equation [24]:

$$\begin{aligned} 1/K_m &= \Sigma K_p + \Sigma K_q \\ &\cong \Sigma K_p \cong \Sigma K_{int} \qquad (\Sigma K_p \gg \Sigma K_q) \\ &= (n-m+1)K_{int} \qquad (n > m) \end{aligned} \tag{4}$$

where n and m represent the dp of the substrate and the number of subsites, respectively. The reciprocal of the Michaelis constant, $1/K_m$, was assumed to be approximated by the sum of the association constant K_{int} for all binding modes in which a substrate molecule occupies all subsites of the enzyme. In other words, the contributions from any partial occupation complexes, either productive or nonproductive, were neglected.

However, the value of K_{int} is not always larger than that of the nonproductive association constants. Indeed, the value of the association constant of lysozyme is almost the same for the nonproductive complex (where the A, B and C subsites are all occupied with the substrate) as that for the productive complex where all subsites (A–F subsites; cleavage between D and E) are occupied [31]. In this case, the contribution to K_q for nonproductive complex(es) is comparable to K_{int} and should be taken into account for the interpretation of K_m as follows: If we assume that $K_q \cong K_{int}$,

$$\begin{aligned} 1/K_m &= (n-m+1)K_{int} + K_q \\ &= [n-(m-1)+1]K_{int} \qquad (K_{int} \cong K_q) \\ &= (n-M+1)K_{int} \qquad (M = m-1) \end{aligned} \tag{5}$$

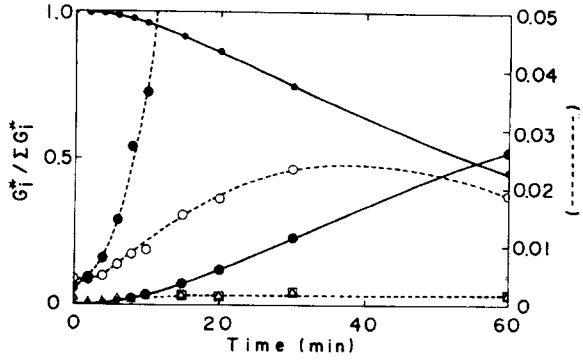

Fig. 2. Time course of various labeled products from the reducing end-labeled maltose (81.6 mM) by BSAm (4.58×10^{-6} M). The reaction was performed in a 0.02 M acetate buffer, pH 5.4 at 25 °C: ●, labeled glucose; •, labeled maltose; ○, G_3^*; △, G_4^*; □, G-G*-G. The amount of labeled saccharides is expressed as the fraction of the total radioactivity. The dotted lines refer to expanded scales (twenty-fold) of that for the solid lines.

As will be seen from the equation, a plot of $\log[1/K_m(n - M + 1)]$ vs. n gives a straight line of constant value when a certain value of M is assumed [14,24]. Shibaoka et al. have obtained the value of four for M (five for the actual subsite number m) [24]. Thus, the discrepancy between the values of Shibaoka et al. and the present study is reasonably understood. Therefore, eq (4) is not always applicable for estimation of the subsite number of endo-amylases.

A lag of time course of products from the reducing end-labeled maltose.—The time course of various products from the reducing end-labeled maltose is shown in Fig. 2. The experiment was performed using 81.6 mM maltose. Highly purified maltose (labeled and unlabeled) was used for this experiment, because commercially obtained maltose contained a small, but significant amount (ca. 1%) of G_3. The addition of 5% maltotriose to substrate maltose suppressed prominently the time lag (an accelerating time course) at the initial stage of the reaction [22]. The decreasing curve of labeled maltose is almost symmetrical to increasing curve of labeled glucose, as seen already in the experiment with unlabeled maltose [22]. The curves showed distinct time lags, which differed from low concentrations of maltose and of higher saccharides (data not shown). Transient formation of the radioactive products higher than the substrate maltose, G_3^* and G_4^*, is shown on the expanded scale (dotted line in Fig. 2). Accumulation of G_3 shows a broad maximum at a significant level of 2% of the initial substrate, then gradually decreases, because G_3 is degraded into glucose and maltose by the subsequent hydrolysis as the reaction proceeds towards the final stage.

Detection of the product G-G-G peculiar to condensation.*—The presence of higher saccharides than the substrate maltose in the hydrolysate suggests the synthetic reactions of BSAm, such as transglycosylation or condensation. In order to discriminate the condensation from other reactions, we tried to determine the amount of maltotriose labeled specifically at the central glucose residue, G-G*-G, in the hydrolysate. When two molecules of maltose are condensed to maltotetraose, G-G*-G-G*, it is cleaved mainly into G-G*-G and G* by BSAm. If present, the cleavage of G_4^* to produce two molecules of maltose results in no net change. In contrast, transglycosylation can produce only the usual reducing end-labeled species. Thus, the product of maltotriose, G-G*-G, is peculiar to the condensation.

The specific maltotriose, G-G*-G, was determined by taking advantage of the characteristic hydrolytic action of beta-amylase from soybean [32]. The enzyme cleaves G_3 exclusively (more than 99.5%) at the first glycosidic bond from the reducing end. Thus, by digestion by beta-amylase, G-G*-G can be determined as radioactive maltose, whereas the end-labeled product G-G-G* yields radioactive glucose. The G-G*-G maltotriose was estimated to be about 8–10% of the total radioactive maltotriose and the remaining of G-G-G* to be about 90%. Therefore, condensation is a minor reaction although not negligible, especially at the initial stage of the reaction. Transglycosylation to produce the G-G-G* was confirmed to be a principal side reaction of BSAm in the degradation of maltose at high concentrations.

Degradation pathway of maltose involving maltosyl transfer.—Based on the above findings, we can depict the following degradation pathway of the poor substrate maltose by BSAm via higher saccharides as intermediate products: First maltose is split to form a glycosyl–enzyme complex releasing a glucose residue at the reducing end, although

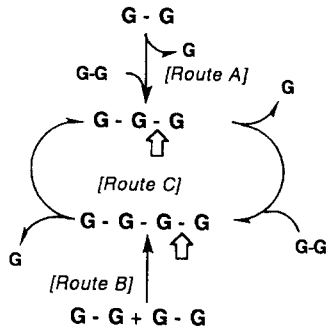

Fig. 3. Scheme for a cyclic pathway for maltose degradation by BSAm. Route A represents a transglycosylation to produce G_3, route B a condensation to produce G_4, and route C a cyclic pathway of transglycosylation and hydrolysis, as a main degradation pathway for maltose. The wide arrow designates a major cleavage point. For details, see text.

the process is very slow. By subsequent transfer process (the primary transfer process), G_3 is produced (route A in Fig. 3), which is mainly cleaved at the first bond to form a maltosyl–enzyme complex, releasing glucose. Maltotriose is still hard to hydrolyze directly, although the hydrolytic rate is much faster than that of maltose (Table 1). Then, from the maltosyl–enzyme complex, G_4 is produced by the addition of another molecule of maltose (the secondary transfer process). Maltotetraose is rapidly hydrolyzed into glucose and G_3 or into two molecules of maltose (Table 1). When the latter case occurs, no net change takes place. It is one of the critical points for the characteristic action pattern of the enzyme that G_3 is produced again in this hydrolytic process of G_4. Then, the maltotriose is used again to form a maltosyl–enzyme complex (route C in Fig. 3).

There is another possible pathway of G_4 formation: two molecules of maltose are condensed to produce G_4 at the initial step (route B in Fig. 3), which is rapidly hydrolyzed into glucose and G_3 (G-G*-G with labeled substrate maltose). Once G_3 is formed either by condensation or transglycosylation, it mediates the degradation of maltose by forming an efficiently circulating cyclic process through transfer action (route C in Fig. 3). In other word, maltose is consumed as acceptor by the glycosyl transfer process with G_3 to form G_4 much more frequently than by direct hydrolysis. Thus, maltotriose behaves as a stimulator or a "catalyst", because it participates in maltose degradation without being consumed during the overall cyclic process. The latent period for accumulation of G_3 is observed as a time lag (an accelerating curve). The side reactions, condensation and transglycosylation, need two molecules of maltose. The rate would be proportional to the square of substrate concentration [15], therefore showing the sigmoidal dependence on the maltose concentration.

Fujimori et al. ran a computer simulation for maltose degradation by the enzyme [22]. Their tentative results, using the kinetic program involving transglycosylation alone as the synthetic process, was in appreciable agreement with their experimental results; the distinct time lag and the sigmoidal dependence. The present study confirmed that their proposal mechanism is essentially correct, except for the condensation reaction. The condensation activity of the enzyme was also found to operate in the degradation of maltose.

Based on the subsite structure of BSAm elucidated, the present study concludes that the principal side reaction of transglycosylation, as an alternative to direct hydrolysis, is degradation of maltose, a poor substrate, thereby causing a sigmoidal concentration dependency and a time lag even in a monomeric enzyme.

References

[1] H. Toda, K. Kondo, and K. Narita, *Proc. Jpn. Acad.*, 58B (1982) 208–212.
[2] B. Svensson, *FEBS Lett.*, 230 (1988) 72–76.
[3] Y. Matsuura, M. Kusunoki, W. Harada, and M. Kakudo, *J. Biochem.*, 95 (1984) 697–702.
[4] G. Buisson, E. Duee, R. Haser, and F. Payan, *EMBO J.*, 6 (1987) 3909–3916.
[5] E. Boel, L. Brady, A.M. Brzozowski, Z. Derewenda, G.G. Dodson, V.J. Jensen, S.B. Petersen, H. Swift, L. Thim, and H.F. Woldike, *Biochemistry*, 29 (1990) 6244–6249.
[6] M. Qian, R. Haser, G. Buisson, E. Duee, and F. Payan, *Biochemistry*, 33 (1994) 6284–6294.
[7] S.B. Larson, A. Greenwood, D. Cascio, J. Day, and A. McPherson, *J. Mol. Biol.*, 235 (1994) 1560–1584.
[8] A. Kadziola, J. Abe, B. Svensson, and R. Haser, *J. Mol. Biol.*, 239 (1994) 104–121.
[9] M. Machius, G. Wiegand, and R. Huber, *J. Mol. Biol.*, 246 (1995) 545–559.
[10] G. Wiegand, O. Epp, and R. Huber, *J. Mol. Biol.*, 247 (1995) 99–110.
[11] J.A. Thoma, G.V.K. Rao, C. Brothers, and J. Spradlin, *J. Biol. Chem.*, 246 (1971) 5621–5635.
[12] J.D. Allen, in D.L. Purich (Ed.), *Methods Enzymol.*, 64 (1980) 248–277.
[13] K. Hiromi, Y. Nitta, C. Numata, and S. Ono, *Biochim. Biophys. Acta*, 302 (1973) 362–375.
[14] Y. Nitta, M. Mizushima, K. Hiromi, and S. Ono, *J. Biochem.*, 69 (1971) 567–576.
[15] T. Suganuma, R. Matsuno, M. Ohnishi, and K. Hiromi, *J. Biochem.*, 84 (1978) 293–316.
[16] T. Suganuma, S. Setoguchi, S. Fujimoto, and T. Nagahama, *Carbohydr. Res.*, 212 (1991) 201–212.
[17] P. Biely, Z. Krátky, and M. Vršanská, *Eur. J. Biochem.*, 119 (1981) 559–564.
[18] C. Seigner, E. Prodanov, and G. Marchis-Mouren, *Biochim. Biophys. Acta*, 913 (1987) 200–209.
[19] I. Matsui, K. Ishikawa, E. Matsui, S. Miyairi, S. Fukui, and K. Honda, *J. Biochem.*, 109 (1991) 566–569.
[20] M. Hrmova, T.P.J. Garrett, and G.B. Fincher, *J. Biol. Chem.*, 270 (1995) 14556–14563.
[21] H. Yamazaki, K. Ohmura, A. Nakayama, Y. Takeichi, K. Otozai, M. Yamasaki, G. Tamura, and K. Yamane, *J. Bacteriol.*, 156 (1983) 327–337.
[22] H. Fujimori, M. Ohnishi, M. Sakoda, R. Matsuno, and K. Hiromi, *J. Biochem.*, 82 (1977) 417–427.
[23] T. Shibaoka, T. Inatani, K. Hiromi, and T. Watanabe, *J. Biochem.*, 77 (1975) 965–968.
[24] T. Shibaoka, K. Miyano, and T. Watanabe, *J. Biochem.*, 76 (1974) 475–479.
[25] J. Fukumoto, T. Yamamoto, and K. Ichikawa, *Proc. Jpn. Acad.*, 27 (1951) 352.
[26] A. Nishida, J. Fukumoto, and T. Yamamoto, *Agr. Biol. Chem.*, 31 (1969) 682–693.
[27] H. Toda and K. Narita, *J. Biochem.*, 63 (1968) 302–307.
[28] J.H. Pazur, *J. Am. Chem. Soc.*, 77 (1955) 1015–1017.
[29] D. French, J.L. Mancusi, M. Abdullah, and G.L. Brammer, *J. Chromatog.*, 19 (1965) 445–447.
[30] K. Ishikawa, I. Matsui, K. Honda, and H. Nakatani, *Biochemistry*, 29 (1990) 7119–7123.
[31] T. Imoto, L.N. Johnson, A.C.T. North, D.C. Phillips, and J.A. Rupley, in P.D. Boyer (Ed.), *The Enzymes*, Vol. 7, Academic Press, New York, 1972, pp 665–868.
[32] T. Suganuma, M. Ohnishi, K. Hiromi, and Y. Morita, *Agr. Biol. Chem.*, 44 (1980) 1111–1117.