Participation of Ions and Solutes on the Thermostability of α-amylase

WANG Yao-Bing[1*] NAGATA Shinichi[2]

[1] (*Institute of Marine Environmental Protection, SOA, P.O.Box* 303, *Dalian* 116023, *China*)

[2] (*Research Center for Inland Seas, Kobe University*, 5-1-1, *Fukae, Higashinada-ku, Kobe* 658-0022, *Japan*)

Abstract Supplement effects of ions, sugars, and amino acids on the thermostability of liquefying type α-amylase from *Bacillus subtilis* were examined. The addition of 1 mmol/L Ca^{2+} or about 50 mmol/L Na^{+} remarkably stimulated the thermostabi-lity of this enzyme among ions examined. The thermostability of the enzyme was enhanced and reduced by the extrinsic addition of 50 mmol/L acidic amino acid such as glutamic acid and alkaline amino acid such as arginine, respectively. With the increases of the concentrations of sugars from 0 to 1000 mmol/L the thermostability of α-amylase increased almost linearly. By the co-existence of Na^{+} or K^{+} with some amino acids or sugars the thermostability of this enzyme was fairly increased. The changes in the fluorescence intensity of α-amylase were examined as a function of the incubation temperature on the enzyme, which showed a good agreement with those of residual activities.

Key words α-amylase, thermostability, ions, compatible solutes, co-existence

Amylases (EC 3.2.1.1) are well known as the end-acting enzymes that hydrolyze starch by cleaving α-1,4-glucosidic linkages randomly, which leads to the production of α-anomer. They are one of the most important commercial enzymes which have wide applications in starch-processing such as brewery, alcohol production, textile, and other industries[1, 2]. The applications of α-amylases, however, have been limited mainly due to the instability faced to a variety of stress environments. Since starch solubilization begins at higher temperatures, α-amylase is required to possess the sufficient heat-tolerant nature for most industrial applications[2, 3]. In this regard, the thermostability of α-amylase is one of the most limited factors for these applications. One possibility to overcome this difficulty is to extract it from the thermophiles which are natively able to tolerate higher temperature atmosphere[4, 5] and the other is to achieve the enhancement of thermostability through the modification of external environments by adding ions or solutes without interfering the stability and biochemical nature of proteins[6, 7].

The salt requirement for halophilic eubacteria seems to be highly variable, depending on the growth conditions such as temperature, pH, kinds and amounts of nutrients available, etc[8]. For instance, the optimum salt concentrations for their growth tend to be high with an increase of incubation temperature[9, 10]. On the other hand, organic small molecules called as compatible solutes which are generally accumulated in the halophilic eubacteria to cope with the external high salinity[11 - 13] are also known as the stabilizers of proteins[14] against the heating, freezing, and drying[15, 16].

From these points of view, it seems to be of interest to examine whether the presence of these solutes as well as ions contributes to the stability of the enzyme or not. Thus, in the present study we examined the changes on the thermostability of α-amylase incubated at 60℃ in the presence of ions and/or solutes through the measurements of the residual activity as well as the fluorescence intensity of the enzyme with the reference to the correlation between them.

1 Materials and Methods

1.1 Enzyme activity

The activity of α-amylase was measured by a modified DNS (3,5-dinitrosalicylic acid) method[17]; 100 μL of enzyme solution, at the final concentration of 5 μg/mL unless otherwise noted, was incubated with 150 μL of soluble starch (4 %) for 1 min at 40℃. After adding 250 μL of DNS stock solution the mixture was boiled for 5 min, and then cooled to 25℃. The absorbance of the samples after dilution was measured at 540 nm using Beckman DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA, USA).

The thermostability of the enzyme was obtained as fol-

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* Corresponding author. Tel: 86-411-4782582; Fax: 86-411-4783336; E-mail: wangyaobing@yip.sina.com

lows; after preheating at 25℃ for 10 min, the mixture consisting of α-amylase solution mentioned above and ions and/or solutes was incubated at 60℃ for 5 min. The thermostability of the enzyme was expressed as the percentage of the residual activity relative to the original one, which was defined as 100 %.

1.2 Measurement of fluorescence

α-amylase solution (5 μg/mL in 10 mmol/L KPi buffer, pH 6.0) was placed in the presence and absence of ions and/or solutes at 60℃ for 5 min. The measurements of fluorescence were carried out using fluorescence spectrophotometer (F-4500, Hitachi Co. Ltd., Japan) under the conditions that the wavelengths were adjusted to 280 and 340 nm as the excitation and emission, respectively. The fraction of protein in the unfolded state (f_U) was calculated according to the equation of $f_U = (y_F - y)/(y_F - y_U)$, where y_F and y_U represent the values of y (fluorescence intensity at a given temperature) characteristic to the folded and unfolded states, respectively[18, 19].

1.3 Chemicals

Liquefying type α-amylase with approximately 95 % purity from *Bacillus subtilis* was purchased from Seikagaku Corp. Ltd., Japan. Ectoine (2-methyl-4-carboxy-3,4,5,6-tetrahydropyrimidine) and its derivatives, hydroxyectoine (2-methyl-4-carboxy-5-hydroxy-3,4,5,6-tetrahydropyrimidine) and homoectoine (2-methyl-3,4,5,6,7-pentahydro-1,3-diazepine), which were of >97 % purity, were kindly provided by Dr. E. A. Galinski, Universität Bonn, Germany. The other chemicals used were of reagent grade or higher grade.

2 Results and Discussion

2.1 Thermostability of α-amylase

The thermostability of α-amylase used was examined in the temperature range from 25℃ to 80℃, fixing the incubation time and the enzyme concentration to 5 min and 5 μg/mL, respectively. As shown in Fig. 1A, the residual activity of the enzyme was almost remained after the incubation at 25 ~ 50℃. Over 55℃, however, it was reduced rapidly; only 10% of the original activity was remained at >65℃. Fixing the incubation temperature at 60℃, the changes in the residual activity of α-amylase were examined as a function of the incubation time. As shown in Fig. 1B, the thermostability of the enzyme was quite sensitive to the incubation time and about 90 % of the original activity was lost after 10 min of incubation. The thermostability of the enzyme as a function of its concentrations was also examined. While the residual activity was about 20 % at the enzyme concentration of 3 μg/mL, it increased to 70 % at 20 μg/mL (data not shown).

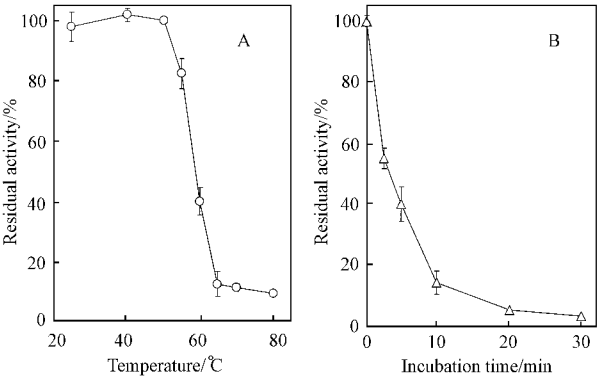

Fig. 1 Thermostability of α-amylase

The residual activity of the enzyme was measured as a function of the incubation temperature, fixing the incubation time for 5 min (A) or as a function of the incubation times, fixing the incubation temperature to 60℃ (B). The values are the averages ± standard deviations from five independent experiments. Details for the activity measurements are described in Materials and Methods

To examine effects of the supplementation of ions or solutes on the enzyme thermostability, based on the preliminary results mentioned above the following conditions: incubation temperature, incubation time, and the enzyme concentration of 60℃, 5 min, and 5 μg/mL, respectively, were adopted, unless otherwise stated.

2.2 Effects of ions, sugars, amino acids and solutes on the thermostability of α-amylase

2.2.1 Ions: The thermostability of α-amylase was examined in the presence of various kinds of cations, among which Ca^{2+} was the most effective thermoprotectant ion, that is, the supplement of 1 mmol/L Ca^{2+} led to a remarkable enhancement of the thermostability (Fig. 2A). The optimal concentration of Ca^{2+} was much lower in comparison with previous studies[4,20], which might be closely related to the fact that α-amylase contains a conserved calcium ion essential to maintain stable and active form of the enzyme[2]. Thus, we analyzed Ca^{2+} contents in the native α-amylase by atomic absorption spectroscopy, and demonstrated the presence of about 8 μmol/L Ca^{2+} corresponding to 8 g-atoms of Ca^{2+} per mole of the enzyme, higher than 2 ~ 5 g-atoms reported by Stein *et al*[21]. It was approved that Ca^{2+} was originally included in the present α-amylase for its maintenance, since the elimination of Ca^{2+} by EDTA treatment led to the dramatic reduction of the activity (data not shown). Similar but smaller effects were observed for Rb^+ and Li^+, the additions of about 2.5 mmol/L of Rb^+ or Li^+ led to the

increase of the thermostability by 1.25 ~ 1.5 folds. The addition of Mg^{2+}, on the other hand, resulted in the reduction of α-amylase thermostability.

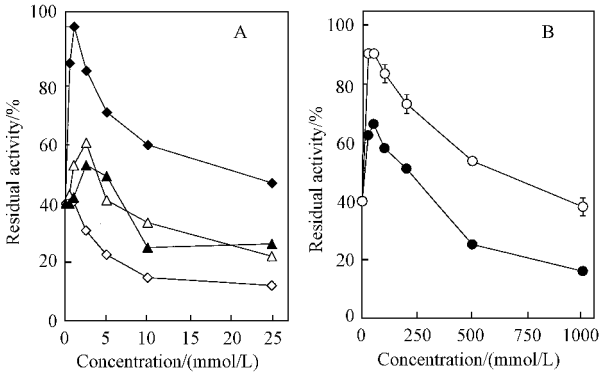

Fig. 2 Supplement effect of cations on the thermostability of α-amylase

The residual activities of the enzyme were measured as a function of $CaCl_2$ (◆), RbCl (▲), LiCl (△), or $MgCl_2$ (◇) concentrations (A) and NaCl (○) or KCl (●) concentrations (B). The values are the averages ± standard deviations from two independent experiments. Details for the activity measurements are described in Materials and Methods

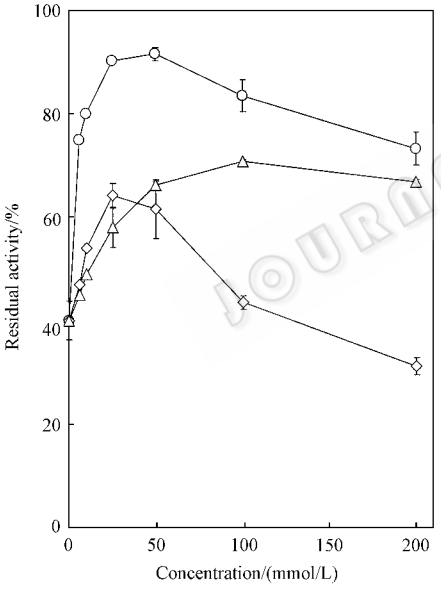

Fig. 3 Supplement effect of anions on the thermostability of α-amylase

The residual activities of the enzyme were measured as a function of NaCl (○), $NaNO_3$ (△), and Na_2SO_4 (◇) concentrations. The values are the averages ± standard deviations from three independent experiments. Details for the activity measurements are described in Materials and Methods

As shown in Fig. 2B, the addition of 25 ~ 50 mmol/L Na^+ or K^+ also led to 2.3 and 1.7-fold stimulation on the thermostability of α-amylases, respectively. However, the addition of higher concentrations of Na^+ or K^+ was compared to Ca^{2+}, Rb^+ and Li^+. Furthermore, we examined the effect of anions such as Cl^-, SO_4^{2-} and NO_3^- as sodium salts, among them the presence of Cl^- afforded the most stimulating effect on the thermostability of the enzyme (Fig. 3).

2.2.2 Sugars: To investigate the effects of sugars on α-amylase thermostability, sorbitol and sucrose were chosen as the representatives of mono- and disaccharides, respectively. Both sugars had remarkable effect on the thermostability enhancement of α-amylase; the residual activity increased almost linearly with the increase of their concentrations, although the addition of sucrose was more efficient than that of sorbitol (Fig. 4). The difference between both sugars might be ascribed to the different numbers of hydroxy groups involved, since the sugar molecules are generally able to bind to the hydrophilic domains of enzymes and change the structure of water surrounding them. Probably due to such interactions the enzyme might be stabilized against the heat denaturation[6].

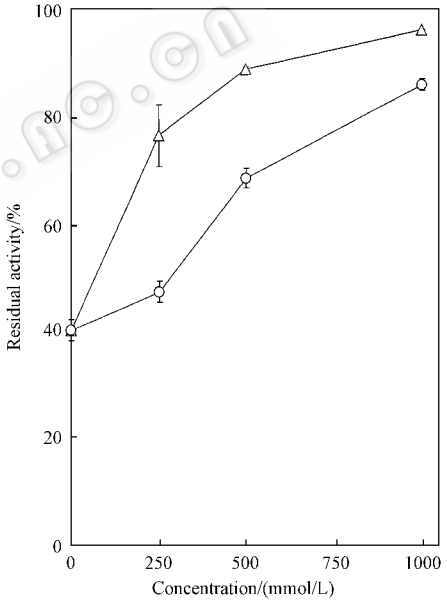

Fig. 4 Supplement effect of sugars on the thermostability of α-amylase

The residual activities of the enzyme were measured as a function of sucrose (△) or sorbitol (○) concentrations. The values are the averages ± standard deviations from three independent experiments. Details for the activity measurements are described in Materials and Methods

2.2.3 Amino acids: An attempt was made to examine the supplement effect of standard amino acids in a same manner mentioned above. As shown in Fig. 5, effects of thermostability enhancement of them on the α-amylase were quite different from one another, and thus they are reasonably divided into three groups: (i) more than 90 % enhancement in the residual enzyme activity was achieved for acidic amino acids such as glutamic acid (Glu) and aspartic acid (Asp). Here it might be meaningful to mention that both

Glu and Asp were used as monosodium salts (Glu-Na or Asp-Na) in this experiment; (ii) in the presence of alkaline amino acids such as arginine, histidine, and lysine, on the contrary, the residual activity was remarkably reduced to less than 10%; (iii) the others mainly consisting of neutral ones had little effect for the thermostability enhancement except for proline, tryptophan, and cysteine, in which >80 % of the residual activity was obtained. These results strongly indicate that the thermostability of α-amylase was stimulated and depressed by anion and cation moieties deduced from acidic and alkaline amino acids, respectively. The reason why the thermostability of α-amylase was reduced by the presence of some alkaline amino acids might be closely related to the inactivation of the enzyme due to the structure deviation through the interaction of them. Thus, it seems to be necessary to examine the structural and functional relationship between them in more detail, for further clarification.

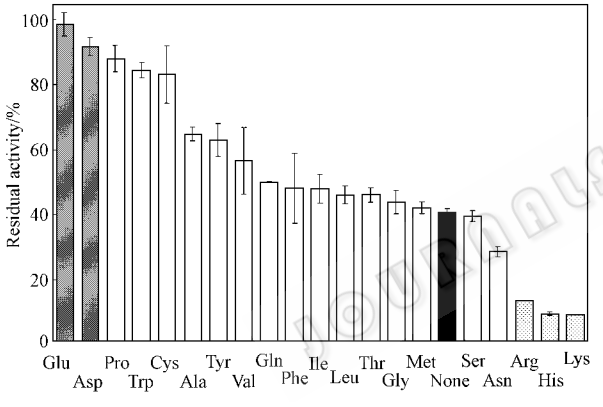

Fig. 5 Supplement effect of standard amino acids on the thermostability of α-amylase

The residual activities of the enzyme were measured when 50 mmol/L of each amino acid was present. The values are the averages ± standard deviations from two independent experiments. Details for the activity measurements are described in Materials and Methods

2.2.4 Compatible solutes: Since ectoine, hydroxyectoine, homoectoine, and glycine betaine (GB) known as the compatible solutes[22] belong to the group of amino acids, the supplement effects of these solutes on the thermostability of α-amylases were also examined (50 ~ 500 mmol/L). As shown in Table 1, the addition of ectoine, hydroxyectoine, and GB showed the slight enhancements of the thermostability, in which at most 1.5 fold stimulation was observed in the presence of 500 mmol/L. The presence of homoectoine, on the other hand, led to the reduction on the thermostability of α-amylase in parallel with the increase of its concentration, suggesting that the unstability of the enzyme might be due to the lack of π-conjugation inside the conformation of homoectoine.

Table 1 Supplement effect of solutes on the thermostability of α-amylase[a]

Solutes[b]	50mmol/L	100mmol/L	200mmol/L	500mmol/L
GB	42.1 ± 1.2	44.2 ± 1.3	45.2 ± 2.3	62.6 ± 0.7
EC	45.0 ± 2.6	45.0 ± 0.7	52.7 ± 3.5	63.6 ± 1.8
HE	40.6 ± 1.5	46.7 ± 1.8	46.1 ± 3.6	62.1 ± 0.9
HoE	46.0 ± 2.2	44.3 ± 2.2	32.2 ± 1.3	—[c]

[a] Values are given as means ± standard deviations from two independent experiments. The residual activity obtained in the absence of solutes was 40 % relative to that without thermal stress.

[b] GB: glycine betaine, EC: ectoine, HE: hydroxyectoine, HoE: homoectoine.

[c] Not detrmined.

2.3 Co-existence of ions and solutes on α-amylase thermostability

In relation to a substantial stimulation of 50 mmol/L Glu-Na or Asp-Na on the enzyme thermostability, further detailed examinations were carried out as a function of these amino acid concentrations up to 1000 mmol/L. As shown in Fig. 6A, the addition of 20 ~ 50 mmol/L of Glu-Na brought about a rapid increase in the thermostability and then almost constant up to 1000 mmol/L. This is quite different from the supplement effect of NaCl, in which the thermostability was linearly reduced in the presence of over 50 mmol/L (Fig. 2B). In contrast, the addition of Asp-Na gave a maximum at 50 ~ 100 mmol/L and then it was decreased almost linearly. In conclusion, Asp-Na might reflect directly the negative effect of Na^+ rather than aspartic acid moiety for the present enzyme, while Glu possesses higher positive response than Na^+ in the form of Glu-Na.

To demonstrate the effect of co-existence of both solutes and cations more clearly, we examined the changes of thermostability in the presence of equimolar GB and Na^+. As shown in Fig. 6B, the residual activity was rapidly increased from 40% to 87 % at 50 mmol/L, and then kept constant until 1000 mmol/L. The behavior was similar to, but a little lower than that of Glu-Na shown in Fig. 6A. In case of GB only, the residual activity showed a gradual increase with the increase of the concentration. Thus, it seems to be of interest to note that Na^+ might assist the active enhancement of the thermostability at the lower concentration, while its own effect was minimized in the presence of GB. In order to obtain the co-existence effect of other cations, we also examined how much enhancement on the thermostability

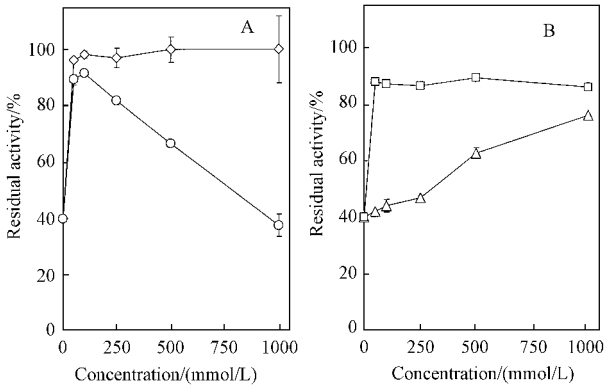

Fig. 6 Thermostability of α-amylase in the co-existence of solutes and Na^+

The residual activities of the enzyme were measured at a various concentrations of monosodium salts of Glu (◇) and Asp (○). (A) It was also done in the presence of GB (△) or equimolar GB and Na^+ (□). (B) Details for the activity measurements are described in Materials and Methods

was achieved when K^+ in place of Na^+ is co-existed with solutes examined above. As a result, the thermostability of α-amylase was not stimulated but reduced to 10% ~ 20% in K^+ and Glu or GB (data not shown). The co-existence of sugars, sucrose or sorbitol, with Na^+ or K^+ led to a slight decrease on the thermostability compared to the absence of each sugar except for the combination of sorbitol and K^+, in which more than 60 % of the activity in their absence was reduced (data not shown).

Remarkable difference between the coexisting effect of some amino acids with Na^+ and K^+ on the thermostability of α-amylase was demonstrated (Table 2). When Na^+ was co-existed with neutral amino acids equimolarly, the thermostability of this α-amylase was stimulated up to > 90 %, although no distinct effect was recognized with the addition of these amino acids only, as shown in Fig. 5. But the stimulation effect was not observed in the co-existence of K^+ and amino acids. In particular, the co-presence of K^+ and alkaline amino acids of lysine or arginine led to a remarkable reduction of thermostability regardless of fairly high degree of activity maintenance in the co-presence of Na^+.

2.4 Correlation between thermostability and protein conformation

To analyze the mechanism on the interaction between solutes and the enzyme, the correlation between the thermostability and the conformation of α-amylase was investigated. The changes in the relative intensity of fluorescence as a function of the incubation temperature were measured at 280 and 340 nm as excitation and emission, respectively. As shown in Fig. 7, the fluorescence intensity was linearly decreased as a temperature raised from 35 to 75℃, suggesting that the unfolded fraction of α-amylase increased with the increase of the incubation temperature based on the equation of thermal kinetics[18].

Table 2 Co-existence effect of standard amino acids and Na^+ or K^+ on the thermostability of α-amylase

Amino acids[a]	Residual activity/%	
	NaCl	KCl
None	90.4	62.5
Glu	98.4	31.6
Asp	91.7	ND[b]
Pro	95.9	52.4
Ala	94.0	65.4
Val	94.6	70.5
Gln	92.9	65.4
Ileu	98.1	75.6
Thr	96.7	61.7
Gly	96.5	68.3
Met	100.0	58.2
Ser	92.6	57.5
Arg	86.2	23.4
His	9.6	3.5
Lys	84.9	41.7

[a] Concentrations of each amino acid and ion were 25 mmol/L. Tryptophan, cysteine, tyrosine, phenylalanine, leucine, and asparagine were omitted due to their undissolveness in the co-exsitence of Na^+ or K^+. [b] Not determined

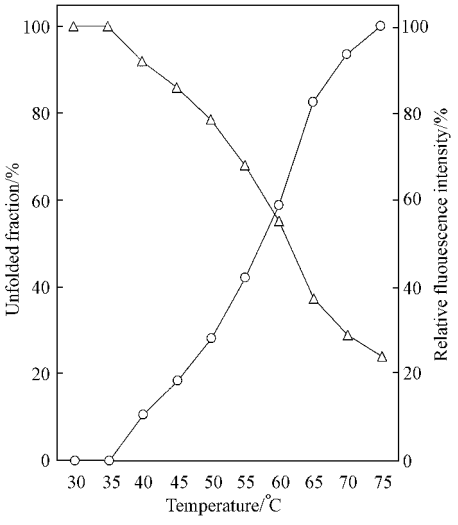

Fig. 7 Unfolding transitions of α-amylase

The changes of unfolding transition (○) and the relative fluorescence intensity (△) of the enzyme were obtained as a function of the incubation temperature. Details for the measurements of fluorescence are described in Materials and Methods

To evaluate the influence of solutes on the conformation of the enzyme, the time dependence of the relative fluorescence intensity was followed in the presence and absence of 25 mmol/L of Na^+ (60℃). In the absence of Na^+, the rela-

tive fluorescence intensity decreased with the incubation time almost linearly (data not shown). In the presence of Na^+, however, it decreased quite gradually and maintained as high as 75 % after heat stress for 30 min although only 20 % of activity was remained without Na^+. Present finding strongly suggests that the presence of Na^+ maintained the conformation of the enzyme against the thermal stress.

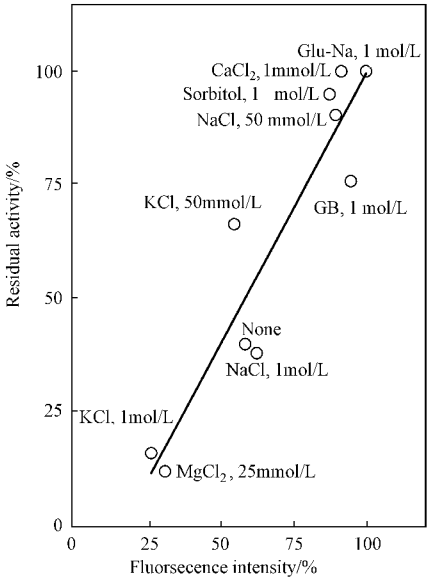

Fig. 8 Relationship between the changes of fluorescence intensity and residual activity of α-amylase

The thermal stability of α-amylase was measured simultaneously by the method of DNS and fluorescence intensity in the absence or presence of solutes or ions, the concentrations of which were indicated. Details for the measurements of thermostability are described in Materials and Methods

Furthermore, we examined the correlation between the changes in fluorescence intensity and the residual activity of the enzyme in the presence of solutes and ions. As shown in Fig. 8, we could obtain a good correlation between them ($r^2 \fallingdotseq 0.88$). For most of enzyme, the loss of enzyme activity in the stressed condition seems to be faster than the change of whole enzyme conformation which is reflected by the measurement of fluorescence, but the measurement of fluorescence intensity may provide direct and reliable tool for the determination of the stability or activity of enzymes. To clarify the correlation of the activity and conformation of enzymes, further study is essential.

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无机离子和有机溶质对 α-淀粉酶热稳定性的影响

王耀兵[1*] 永田进一[2]

[1](国家海洋局海洋环境保护研究所,大连 116023)

[2](神户大学 内海域環境教育研究センタ 環境生化学研究分野 日本神户市东滩区深江南町 5-1-1)

摘 要 长期以来,如何提高酶蛋白的热稳定性是分子生物学、生物工程学、化学工业等所关注的重要研究课题之一。分析了多种无机离子、糖和氨基酸对枯草杆菌液化型 α-淀粉酶热稳定性的影响以及它们的共存效应,获取了一些对相关研究领域具有理论参考和实际应用价值的实验结果。

在无机盐中,1mmol/L 的钙离子或 50mmol/L 的钠离子能显著地提高该酶的热稳定性 酸性氨基酸和碱性氨基酸表现出相反的结果 酸性氨基酸具有明显的增强作用,碱性氨基酸却使之降低 随着糖浓度的增加(0 ~ 1000mmol/L),该淀粉酶的热稳定性呈线性增高 当钠离子或钾离子与某些氨基酸或糖类共同存在时,对该淀粉酶的热稳定性表现出了明显的协同作用。试图通过检测酶蛋白分子荧光强度改变来反映该酶的热稳定性变化,其结果是 随着温度的改变,酶蛋白的荧光强度的衰减与残余酶活性之间显示了良好的相关性。从而说明热环境使酶蛋白分子的螺旋结构发生变化而失活,某些溶质的存在可能是通过作用于蛋白质分子的立体结构而影响该酶的热稳定性。

关键词 α-淀粉酶,热稳定性,离子,补偿溶质,共存

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* **通讯作者**。Tel:86-411-4782582 (13304119231); Fax:86-411-4783336; E-mail:wangyaobing@vip.sina.com