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Purification and Identification of the Essential Ionizable Groups of Honeybee, *Apis mellifera* L., Trehalase

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Trehalase (EC 3.2.1.28) of the bound type was purified as an electrophoretically homogeneous protein from adult honeybees by fractionation with ammonium sulfate, hydrophobic chromatography, and DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, butyl-Toyopearl 650M, and *p*-aminophenyl β-glucoside Sepharose 4B column chromatographies. The enzyme preparation was confirmed to be a monomeric protein containing 3.1% carbohydrate. The molecular weight was estimated to be approximately 69,000, and the optimum pH was 6.7. The Michaelis constant (K_m) was 0.66 mM, and the molecular activity (k_0) was 86.2 s^{-1}. The enzyme was an "inverting" type which produced β-glucose from α, α-trehalose. Dependence of the V and K_m values on pH gave values for the ionization constants, pKe_1 and pKe_2, of essential ionizable groups 1 and 2 of the free enzyme of 5.3 and 8.5, respectively. When the dielectric constant of the reaction mixture was decreased, pKe_1, and pKe_2 were shifted to higher values of +0.2 and +0.5 pH unit, respectively. The ionization heat (ΔH) of ionizable group 1 was estimated to be +1.8 kcal/mol, and the ΔH value of group 2 was +1.5 kcal/mol. These findings strongly support the notion that the essential ionizable groups of honeybee trehalase are two kinds of carboxyl groups, one being a dissociated type (-COO^-, ionizable group 1) and the other a protonated type (-COOH, ionizable group 2), although the pKe_2 value is high.

Key words: honeybee trehalase; α,α-trehalose; α,β-trehalose; essential ionizable group; *Apis mellifera*

Trehalase (α,α-trehalase, glucohydrolase, EC 3.2.1.28) is an anomer-inverting (α→β) glycosidase catalyzing the hydrolysis of the α-glucosidic *O*-linkage of α,α-trehalose to release β-glucose. The substrate specificity of trehalase is extremely high and specific to only α,α-trehalose in natural sugars, although a synthetic α-glucosyl fluoride[1] is known to be exceptionally hydrolyzed to release β-glucose, and the double bond of synthetic D-*gluco*-octenitol[2] to be hydrated to give 1,2-didioxy-D-*gluco*-octulose by trehalase.

Trehalase is widely distributed in microorganisms,[3,4] plants,[5,6] and invertebrate[7,8] and vertebrate animals.[9–11] However, the physiological functions of trehalase *in vivo* have remained obscure. In insects, the soluble form and bound form of trehalases have been respectively localized in the hemolymph and tissues. Since the blood sugar of insects is generally known to be mainly trehalose, although glucose and fructose have also been observed to be blood sugar constituents in the honeybee, trehalase in the hemolymph is assumed to supply glucose by splitting trehalose.

In respect of European honeybee (*Apis mellifera* L.) trehalase, Lefebvre and Huber[12] first reported the occurrence of soluble and bound types of trehalase. They have, however, stated that there was no marked difference between the properties, for instance, M_r 65,000 and optimum pH 6.5, of the two forms of trehalase purified from the honeybee thorax.[13]

The amino acid sequences of trehalases from *Escherichia coli*,[14] rabbit small intestine,[15] and silkworm pupal midgut (*Bombyx mori*)[16] have been recently revealed by nucleotide sequence analyses. However, the ionizable groups essential to the catalytic activity have not been elucidated. In trehalase from the lady beetle (*Harmonia axyridis*),[17] cockchafer (*Melolontha vulgaris*),[18] and silkworm,[16] the possibility has been suggested that the imidazole group of the histidyl residue is an essential ionizable group for the catalytic reaction, but the suggestion should be reinvestigated because an essential ionizable group having a high pKe value seems not always to be an imidazole group. We are interested in the relationship between the catalytic reaction mechanism and the structure of the active site of trehalase,

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so an analysis of the primary structure of honeybee trehalose is in progress.

This paper describes the purification of honeybee trehalase, and the identification of essential ionizable groups directly involved in the catalytic reaction of trehalase.

Materials and Methods

Enzyme source. Honeybees (*Apis mellifera* L.) were purchased from a local farm in Hokkaido (Ishikari, Japan) and kept in a hive from June to October on the campus of Hokkaido University. After keeping, the bees were frozen and stored at −40°C for the experiments.

Materials. Trehalose (α,α-trehalose) and arabitol were purchased from Nacalai Tesque Inc. (Kyoto, Japan); α-glucose, from Wako Pure Chemical Ind. Ltd. (Osaka, Japan); and β-glucose, from Sigma Chemical Co. (St. Louis, USA). α,β-Trehalose (neotrehalose) was kindly supplied by National Food Research Institute (Tsukuba, Japan); and 6-α-glucosyl-trehalose and 6-β-glucosyl-trehalose, by Meiji Institute of Health Science (Odawara, Japan). The trimethylsilylation reagent (TMSI-H) containing hexamethyl disilazane and trimethylchlorosilane in pyridine were purchased from Gasukuro Kogyo Co. Ltd. (Tokyo, Japan); and 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide and diethylpyrocarbonate, from Nacalai Tesque Inc. *p*-Aminophenyl β-glucoside-Sepharose 4B was synthesized by the method of Block and Burger.[19]

Enzyme assay. Trehalase activity was determined by the glucose oxidase method[20] with our modification, using "Glucose AR-II" (Wako Pure Chemical Ind. Ltd.). The standard reaction mixture containing 0.2 ml of 0.5% trehalose, 0.2 ml of 0.1 M sodium phosphate buffer (pH 6.75), and 0.1 ml of the enzyme solution in a total 0.5 ml volume was incubated at 35°C. One unit of the enzyme activity is defined as the amount of enzyme which hydrolyzed 1 μmole of trehalose per minute under the conditions just described.

Protein determination. The concentration of trehalase was determined spectrophotometrically on the basis of the value that $E^{1\%}_{1cm}$ at 280 nm was 18.6, which was obtained from the relationship between the dry weight and the optical density[21] for the purified enzyme preparation. In the purification steps, however, the coloring matter-binding method,[22,23] using Coomassie Brilliant Blue G-250 (CBB) and measurement on the basis of the assumption that $E^{1\%}_{1cm}$ at 280 nm was 10, was also used.

Carbohydrate determination. The sugar content of the enzyme preparation was estimated as glucose by the phenol-sulfuric acid method.[24]

Hydrophobic chromatography. Chromatography by ammonium sulfate solubilization was carried out by the same procedure as that described in our previous paper[25] according to King's report.[26]

Gas-liquid chromatography (GLC). GLC of anomeric forms of the sugars was conducted by the novel quantitative method described in a previous paper.[27]

Electrophoresis. Electrophoresis on acrylamide gel (7.5%)[28] was carried out in a β-alanine-acetic acid buffer (pH 4.0) with a current of 3 mA per tube (0.5 × 7 cm) for about 120 min at 4 to 5°C, using cytochrome *c* as the marker. Protein was stained with CBB.

Sodium dodecylsulfate (SDS)-polyamide gel-electrophoresis was done by Laemmli's method.[29] Purified trehalase was diluted 1:1 in the sample buffer (4% SDS, 0.02% BPB, 20% glycerol, 10% 2-mercaptoethanol, and 125 mM Tris-HCl at pH 6.8) and heated for 5 min at 95°C. The sample was loaded on 4% acrylamide stacking gel (pH 6.8) at 15 mA for 15 min, and then electrophoresed on 8.0% acrylamide (pH 8.8) at 25 mA for 50 min (Mini PROTEAN II electophoresis chamber, Bio-Rad). Protein was detected with Rapid CBB KANTO (Kanto Kagaku Co., Tokyo, Japan).

Results and Discussion

Purification of honeybee bound trehalase

The frozen honeybees (500 g) were mechanically ground with a small amount of M/30 sodium phosphate buffer (pH 6.3) in a porcelain mortar. To the homogenate was added the same buffer in a final volume of about 3,000 ml. After being kept overnight while stirring at 4°C, the suspension was centrifuged at 15,000 × *g* for 15 min, and the resulting precipitate was suspended in 500 ml of the same buffer. The suspension was centrifuged again at 15,000 × *g* for 15 min. To remove the soluble trehalase, the same procedure was repeated 3 times until no trehalase activity was detectable in the supernatant.

The resulting precipitate was suspended in 500 ml of M/30 sodium phosphate buffer (pH 6.3). To solubilize the bound trehalase from the precipitate, the pH of the suspension was adjusted to 8.0 with M/10 sodium hydroxide according to the report of Tolbot *et al.*,[8] and kept overnight while stirring at 4°C. After centrifugation to remove the insoluble materials at 15,000 × *g* for 60 min, the supernatant adjusted to pH 6.3 with N/10 hydrogen chloride was dialyzed against M/30 sodium phosphate buffer (pH 6.3). The dialyzed solution was used as the crude ex-

tract (1,000 ml) for the purification of trehalase.

To the dialyzed solution was slowly added solid ammonium sulfate up to 65% saturation while stirring, and the turbid solution was allowed to stand overnight at 4°C. The resulting precipitate was collected by centrifugation at 15,000×*g* for 60 min and then dissolved in 200 ml of an M/100 sodium phosphate buffer (pH 6.5). After the solution had been throughly dialyzed against the same buffer, the insoluble material was centrifugally removed at 10,000 ×*g* for 15 min.

The dialyzed solution (2,700 ml) was applied to a DEAE-Sepharose CL-6B column (3.6×49 cm) that had been equilibrated with the M/100 sodium phosphate buffer (pH 6.5). Linear gradient elution was performed by increasing the concentration of sodium chloride (0 to 0.4 M) in the same buffer, the flow rate was 180 ml/h and each fraction, 12 ml. The active fractions (nos. 260 to 272) were collected and dialyzed against the M/20 sodium acetate buffer (pH 5.0).

The dialyzed solution (540 ml) was subjected to chromatography in a CM-Sepharose CL-6B column (2.6×37 cm) that had been equilibrated with the M/20 sodium phosphate buffer. The enzyme was eluted with a linear gradient of sodium chloride (0 M to 0.2 M) in the same buffer, the flow rate was 16 ml/h up to fraction no. 50 (16 ml/fraction), and then 20 ml/h (8 ml/fraction). The active fractions (nos. 103 to 112) were combined and dialyzed against M/10 sodium phosphate buffer (pH 7.0). To the dialyzed solution was added solid ammonium sulfate up to 30% saturation.

The solution was chromatographed in a butyl-Toyopearl 650M colum (1.6×10 cm) that had been equilibrated with an ammonium sulfate solution of 30% saturation in the M/10 sodium phosphate buffer, pH7.0. As shown in Fig. 1, hydrophobic elution[25,26] was carried out by linearly decreasing the concentration of ammonium sulfate from 30% to 5% saturation.

The active fractions (nos. 54 to 70) were collected and dialyzed against M/100 sodium phosphate buffer (pH 7.0), and then chromatographed again in the DEAE-Sepharose CL-6B column (1.6×10 cm) that had been equilibrated with the M/100 sodium phosphate buffer (pH 7.0) in a similar manner to that already described; a linear gradient of sodium chloride (0 to 0.3 M) was used at a flow rate of 9 ml/h (1.5 ml/fraction).

The active fraction (nos. 135 to 150) were recovered and dialyzed against M/100 sodium phosphate buffer (pH 6.0), and then chromatographed in a *p*-aminophenyl *β*-glucoside-Sepharose 4B column that had been equilibrated with the same buffer as shown in Fig. 2. The eluate of fraction nos. 47 to 54 was pooled and used as purified honeybee "bound trehalase" as tentatively designated in this paper.

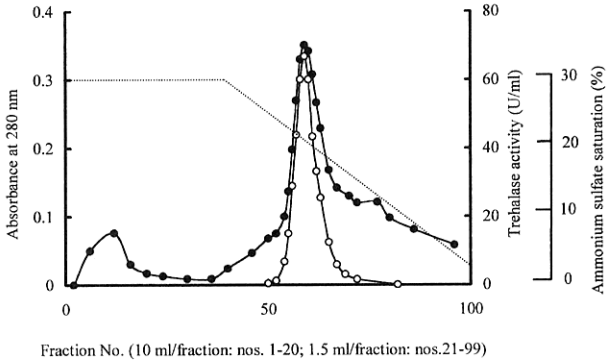


Fig. 1. Chromatography of Honeybee Bound Trehalase on Butyl-Toyopearl 650M Column.

●, Absorbance at 280 nm; ○, trehalase activity; ···, ammonium sulfate-saturation; column size, 1.6×10 cm; sample, 100 ml; equilibrium, 30% ammonium sulfate-saturated M/10 sodium phosphate buffer (pH 7.0); elution, 30–0% ammonium sulfate; and flow rate, 15 ml/h (nos. 1–20) and 25 ml/h (nos. 21–99).

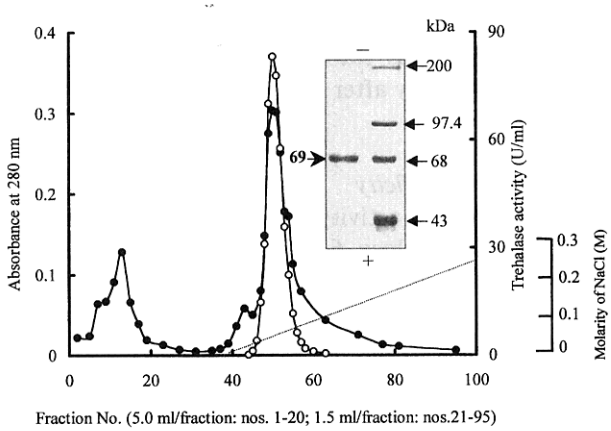


Fig. 2. Chromatography of Honeybee Bound Trehalase on *p*-Aminophenyl *β*-Glucoside Sepharose 4B Column.

●, Absorbance at 280 nm; ○, trehalase activity; ···, molarity of NaCl; column size, 1.6×15 cm; sample, 30 ml; equilibrium, M/100 sodium phosphate buffer (pH 6.0); elution, NaCl; and flow rate, 5 ml/h. Inset, SDS-polyarylamide gel electrophoresis; molecular weights of trehalase and marker proteins: 69,000 for trehalase, 200,000 for myosin (H-chain), 97,400 for phosphorylase *b*, 68,000 for bovine serum albumin, and 43,000 for ovalbumin.

The enzyme preparation migrated as a single protein band by disc polyacrylamide gel electrophoresis. SDS-disc polyacrylamide gel electrophoresis indicated the enzyme to be a monomeric protein, and its molecular weight was estimated to be approximately 69,000 by a comparison with marker proteins (Fig. 2), although it was estimated to be 62,000 by gel chromatography with a Bio-Gel P-150 column. The purification procedures for trehalase are summarized in Table 1. The value of $E^{1\%}_{1cm}$ at 280 nm was 18.6, and the carbohydrate content in the enzyme was 3.1% as glucose.

Effects of pH and temperature

The effect of pH on the activity of trehalase was ex-

Table 1. Summary of the Purification Procedure for Honeybee Trehalase

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude extract	9590*	764	0.0797	100	1
65% Ammonium sulfate precipitate	8700*	739	0.0849	99.1	1.07
DEAE-Sepharose CL-6B	560	720	0.0777	97.4	9.15
CM-Sepharose CL-6B	26.5	641	24.2	83.9	304
Butyl-Toyopearl 650M	5.39	635	118	83.1	180
DEAE-Sepharose CL-6B	3.32	612	184	80.1	2310
p-Aminophenyl β-Glucoside Sepharose-4B	2.56	589	230	77.1	2890
	(1.38)		(427)		(5360)

* These values were measured by the coloring matter-binding method,[22,23] using CBB.
The values in parentheses were measured on the basis of the fact that $E^{1\%}_{1cm}$ at 280 nm was 18.6, and the other values, on the basis of the assumption that $E^{1\%}_{1cm}$ at 280 nm was 10.

amined by using the purified trehalase. As shown in Fig. 3, the optimum pH value for the enzyme was 6.7. The enzyme was stable in the range from pH 4.5 to 12.5, and appeared to be more stable in the acidic than alkaline region.

The enzyme was stable up to 40°C, but lost its activity completely after being incubated at 60°C for 15 min.

Substrate specificity

The hydrolytic activity toward α,α-trehalose (trehalose), α,β-trehalose, 6-α-glucosyl-trehalose, and 6-β-glucosyl-trehalose was checked. Only α,α-trehalose was hydrolyzed by the enzyme, implying that the substrate specificity of trehalase was very high. The effect of the concentration of trehalose on the rate of hydrolysis was next examined. Figure 4 shows Lineweaver-Burk plot for hydrolysis of trehalose; the Michaelis constant (K_m) was 0.66 mM, and the molecular activity (k_0) was 86.2 s^{-1} at 35°C.

Anomeric form of glucose produced from trehalose

The anomeric form of glucose liberated from trehalose by honeybee trehalase was examined by GLC.[27] The chromatograms are shown in Fig. 5. Equimolecular quantities of α- and β-glucose were detected as the products from trehalose. This implies that trehalase is an "inverting" enzyme to liberate the product inverted (α to β) the anomeric configuration of the substrate in the hydrolytic reaction.

Determination of anomeric configuration of the product is significant for understanding the reaction mechanism of glycosidases. In respect of the product anomer of trehalase, Labat *et al.*[30] have reported that pig kidney trehalase was an α-glycosidase acting with retention of the α-anomeric configuration to produce α-glucose from trehalose. However, Defaye *et al.*[18] have reported that trehalase from the cockchafer (*Melolontha vulgaris*) catalyzed the inversion of the product anomer in the hydrolytic reaction. Clifford[31] has also clarified an inversion of the configuration at C_1 of trehalose in the hydrolytic reaction by the flesh

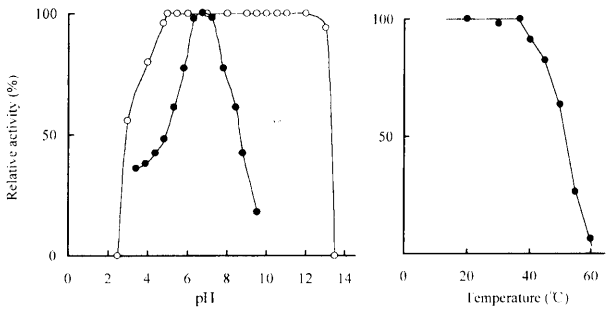


Fig. 3. pH-Activity and -Stability Curves, and Temperature-stability Curve of Honeybee Trehalase.

pH-Activity curve (● in left panel): A reaction mixture containing 0.4 ml of 0.5% trehalose, 0.4 ml of a Britton-Robinson buffer of various pH values (a mixture of 40 mM acetic acid, 40 mM phosphoric acid, and 40 mM boric acid whose pH was adjusted by 200 mM NaOH) and 0.2 ml of the enzyme solution was incubated at 35°C for 5 min.

pH-Stability curve (○ in left panel): After a mixture containing 0.01 ml of the enzyme solution, 0.1 ml of a Britton-Robinson buffer of various pH values and 0.01 ml of 1% Triton X-100 had been incubated at 4°C for 24 h, 0.33 ml of M/2.5 sodium phosphate buffer (pH 6.7) and 0.05 ml of 2% trehalose were added, and the reaction mixture was incubated at 35°C for 5 min.

Temperature-stability curve (● in right panel): After a mixture containing 0.05 ml of the enzyme solution, 0.2 ml of M/10 sodium phosphate buffer (pH 6.7) and 0.05 ml of 0.1% Triton X-100 had been treated at various temperatures for 15 min, 0.2 ml of 0.5% trehalose was added, and the residual activity was measured at 35°C for 10 min.

fly (*Sarcophage barbata*). Later, Nakano *et al.*[32] verified that pig kidney trehalase was an inverting α-glycosidase to release β-glucose from trehalose. These findings indicate that trehalase is an "inverting" enzyme of anomeric configuration, as well as glucoamylase.

Figure 5(E) shows the GLC trace for honeybee blood sugars. Besides trehalose, α-and β-glucose and fructose were detected as blood sugars in the haemolymph; the peak of α-glucose overlapping that of fructose appears higher than that of β-glucose.

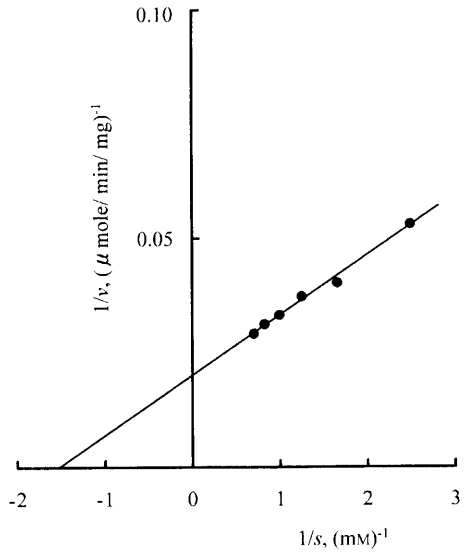


Fig. 4. Lineweaver-Burk Plot for the Hydrolysis of Trehalose by Honeybee Trehalase.

The initial rate was measured at 35°C in a reaction mixture containing 0.2 ml of a substrate solution of various concentrations in M/10 sodium phosphate buffer (pH 6.7) and 0.05 ml of the enzyme solution (2 μg of trehalase).

Identification of the essential ionizable groups for the hydrolytic reaction

The ionizable groups essentially involved in catalytic activity of carbohydrate-hydrolases are generally considered to be carboxyl, -COOH, in the alkaline region, and carboxylate, $-COO^-$, in the acidic region.[33] In the enzyme having optimum pH in the neutral region, however, the possibility of an imidazole group can't be completely ruled out.[34]

The catalytic ionizable groups of trehalase were investigated by chemical modification with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (water-soluble carbodiimide, WSC) to specifically react with the carboxyl or carboxylate group, and with diethyl pyrocarbonate (DEPC) that is specific for the imidazole group. While the data are not shown, honeybee trehalase was inactivated with both WSC and DEPC. In the chemical modification, the essential ionizable group in the acidic region seemed to be carboxylate ($-COO^-$), but we could not elucidate whether the essential ionizable group in the alkaline region was a carboxyl or imidazole group.

Therefore, a kinetic analysis by the Dixon-Webb semi-logarithmic plots, pK_m, log V, and log (V/K_m), was attempted to identify the essential ionizable groups of the enzyme. Honeybee trehalase was found to be strongly inhibited by such cations as sodium, potasium and ammonium (data not shown) and also by TEMED which was a constituent of the buffer in this experiment (Fig. 6). The inhibitory effect was high, especially in the alkaline region (Fig. 6), and the activity was also significantly dependent on the buffer concentration. There was little or no effect at

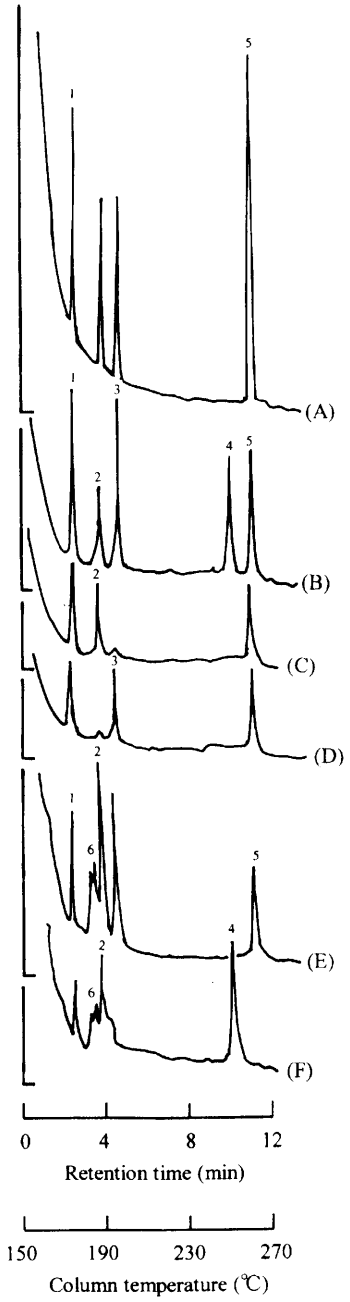


Fig. 5. Gas-Liquid Chromatograms of the TMS Derivatives of Products from Trehalose by Honeybee Trehalase (A) and Blood Sugars (E) of Honeybee.

A reaction mixture consisting of 0.03 ml of 0.5% trehalose in M/20 sodium phosphate buffer (pH 6.0) and 0.02 ml of the trehalase solution (0.09 μg) was incubated for 1 min. The reaction mixture was immediately frozen by immersing the test tube in dry ice-acetone, and then lyophilized at −50°C. The freeze-dried sample was converted to the trimethylsilyl (TMS) compound by incubating with 0.05 ml of a TMSI-H reagent at 80°C for 10 min, and then subjected to gas-liquid chromatography.[27] Haemolymph collected from the honeybee body by a glass capillary was lyophilized and used as the blood sugar sample. The TMS derivative of arabitol was used as the internal standard. The column temperature was elevated at a rate of 10°C/min from 150 to 270°C. (A) arabitol (1) and products (2 and 3) from trehalose (5); (B) equilibrated α-glucose (2), β-glucose (3), sucrose (4), and trehalose (5); (C) α-glucose (2) and trehalose (5); (D) β-glucose (3) and trehalose (5); (E) sugars in haemolymph; (F) α-glucose (2), fructose (6), and sucrose (4).

pH 6.7 or below 50 mM (Fig. 6). In the alkaline region of more than pH 6.7, however, the rate parameters should be determined without the influence of pH or ions on the rate of the enzyme reaction. Figure 7 shows a typical example of the experimental results measured at pH 7.5; this shows the intrinsic K_m and V values without the effect of pH or the concentration of buffer. Each intercept on the ordinate in Fig. 7(A) indicates the reciprocal of the rate (v) of hydrolysis of trehalose independently of the buffer concentration. In Fig. 7(B), the values are plotted against the reciprocal for the concentration of trehalose on the abscissa. The K_m and V values at pH 7.5 were obtained from Fig. 7(B). In the alkaline region of more than pH 7.0, all of the K_m and V values were determined in the same manner.

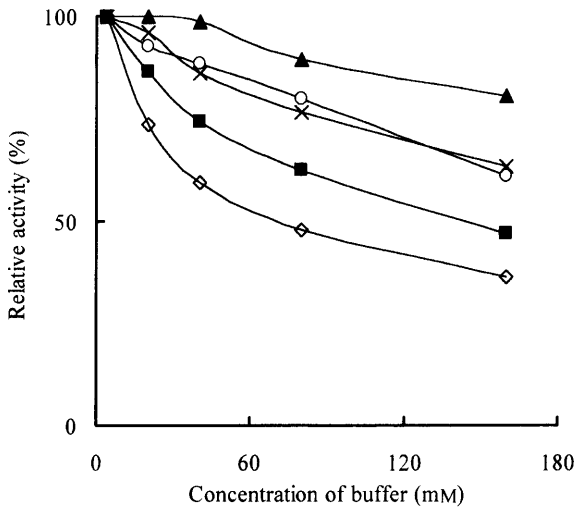


Fig. 6. Effects of pH and Concentration of the Imidazol-TEMED-HCl Buffer on the Inhibition of Trehalase.

A mixture containing 0.1 ml of the trehalase solution, 0.2 ml of 0.5% trehalose and 0.2 ml of a buffer solution at various pH values was incubated at 35°C for 5 min.

▲, pH 6.7; ○, pH 7.0; ×, pH 7.5; ■, pH 8.0; ◇, pH 8.5.

Figure 8 shows the Dixon-Webb semi-logarithmic plots,[35] the relationship between pH and the rate parameters of the $pK_m(=-\log K_m)$, $\log V$, and $\log (V/K_m)$ values. The pK_m value is almost constant, but $\log V$ and $\log (V/K_m)$ both decrease in the acidic and alkaline regions. The p*Ke* values of ionizable group 1 in the acidic region and of ionizable group 2 in the alkaline region of the free enzyme were estimated from the plots of $\log V$ and $\log (V/K_m)$ *versus* pH. The pKe_1 of 5.3 and the pKe_2 of 8.5 were obtained from the pH values corresponding to the points indicated by the guide lines (dashed lines) in Fig. 8. The pKe_1 value suggests that the essential ionizable group was carboxylate ($-COO^-$) in the acidic region.

The heat of ionization (ΔH) is important for identifying the ionizable group. The ΔH value was calculated from the effect of temperature dependent on p*Ke* by using the van't Hoff's equation:[35]

$$d(\mathrm{p}Ke)/d(1/T) = \Delta H/2.303R$$

where T and R are the absolute temperature and gas constant, respectively. A plot of p*Ke* *versus* $1/T$ should give a straight line with a slope of $\Delta H/2.303R$. The plots are shown in Fig. 9, from which ΔH for ionizable group 1 and ΔH_2 for ionizable group 2 were calculated to be +1.8 kcal/mol and +1.5 kcal/mol, respectively. The p*Ke* value for ionizable group 2 implies an imidazole group, but also a carboxyl group of high p*Ke* value which can be shifted by interaction with vicinal amino acid residues in the active site. Since the ΔH value for the carboxyl group is about ±1.5 kcal/mol, and that for

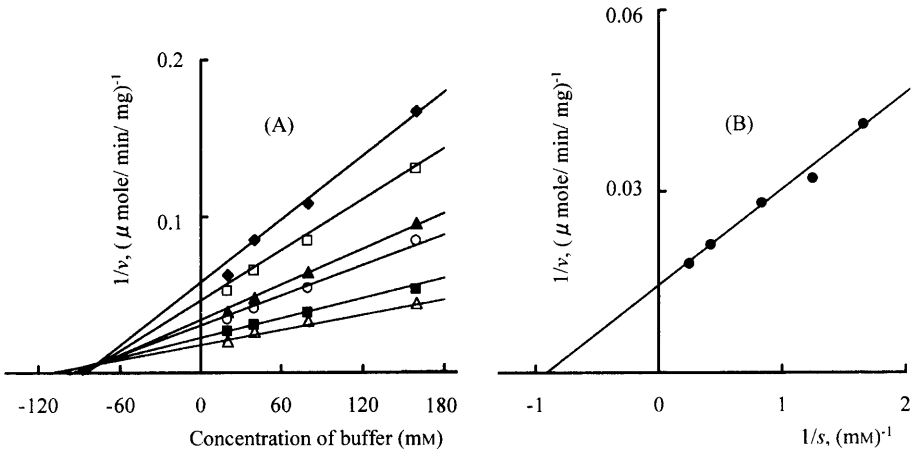


Fig. 7. Reciprocal Plots of the Initial Rate *versus* Buffer Concentration (A) and a Lineweaver-Burk Plot (B) for the Hydrolysis of Trehalose by Honeybee Trehalase.

A mixture containing 0.2 ml of an imidazol-TEMED-HCl buffer (pH 7.5) at various concentrations, 0.1 ml of the trehalase solution (0.01 μg) and 0.1 ml of 0.4 to 4.0 mM trehalose was inhibited at 35°C for 5 min.

The symbols in (A) show the concentration of trehalose.

◆, 0.4 mM; □, 0.6 mM; ▲, 0.8 mM; ○, 1.2 mM; ■, 2.4 mM; △, 4.0 mM.

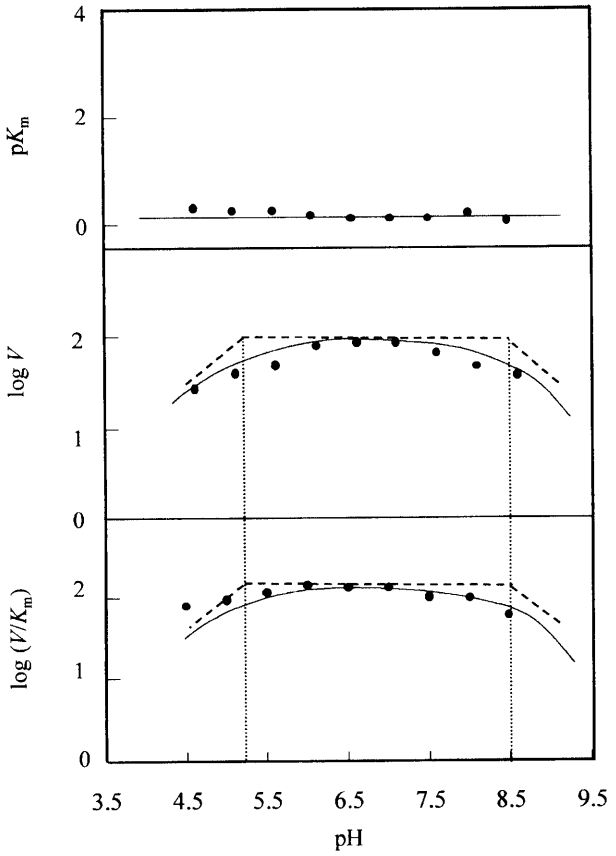


Fig. 8. Dependence of K_m, V, and V/K_m Values on pH for the Hydrolysis of Trehalose by Honeybee Trehalase.

The kinetic constants, K_m and V, were estimated from the Lineweaver-Burk plot at different pH values. K_m, mM; V, μmole of trehalose/mg of protein/min.

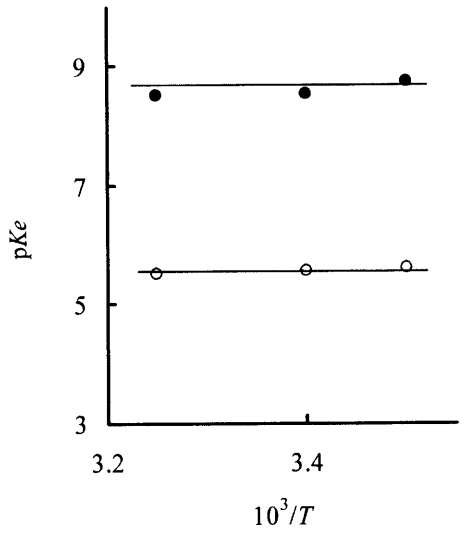


Fig. 9. Dependence of pKe_1 and pKe_2 on Temperature for the Hydrolysis of Trehalose.

pKe at different temperatures was estimated from the Dixon-Webb logarithmic plot.

○, pKe_1; ●, pKe_2.

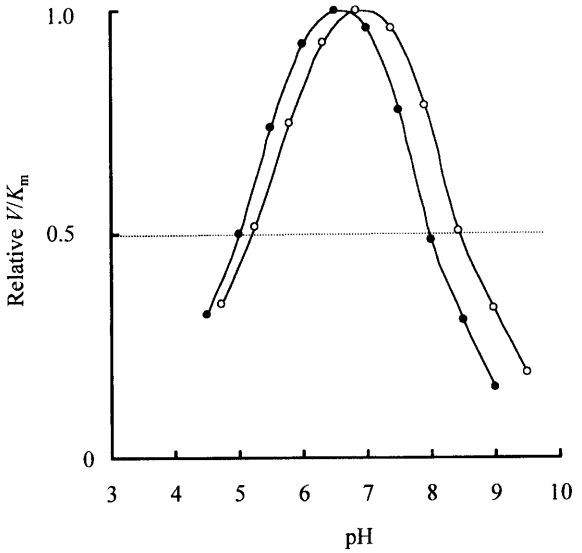


Fig. 10. Effect of Methanol on the Hydrolysis of Trehalose by Honeybee Trehalase.

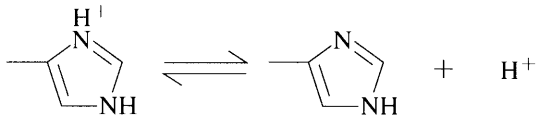
A reaction mixture containing 0.2 ml of an imidazol-TEMED-HCl buffer, 0.1 ml of the trehalase solution (0.08 μg), 0.1 ml of trehalose, and 0.1 ml of methanol (or water) was incubated at 35°C for 5 min. ○, in the presence of 20% methanol; ●, in the absence of methanol.

Since the initial rate of hydrolysis in the alkaline region depended on the pH and buffer concentration (see the text), the K_m and V values at individual pH values were calculated from the data in Fig. 7.

the imidazole group is about +7.5 kcal/mol,[35] however, the ΔH_2 value being less than 2 kcal/mol suggests that essential ionizable group 2 is carboxyl (-COOH). This notion is supported by the shift of pKe to a higher value in the medium with a low dielectric constant, as described next.

The effect of the dielectric constant of the solvent on pKe provides important information on the ionizable group.[35] Methanol (20%) was used to decrease the dielectric constant in the reaction mixture. The relative V/K_m values at various pH values in the absence and presence of 20% methanol are plotted in Fig. 10, where the pKe values corresponding to the pH values are a half of the V/K_m value at optimimum pH in the absence of methanol. Both the pKe values increased in 20% methanol, shifting +0.2 and +0.5 pH unit for pKe_1 and pKe_2, respectively. This shift of pKe_2 to a higher value in the medium with a low dielectric constant implies that ionizable group 2 also is carboxyl (-COOH), because an ionizable group which increases its net charge in dissociation such as the carboxyl group (Scheme 1) can be expected to suppress charge separation in the medium. On the other hand, the total change in ionization in the case of the imidazole group (Scheme 2) would be little affected by a change in the dielectric constant.[35] In the case of lady beetle trehalase,[17] the catalytic amino acid residue was assumed to be a histidyl residue only by chemical modification with DEPC and by the pKe

Scheme 1



Scheme 2

values. However, checking the ΔH value and the effect of the dielectric constant of the solvent are both considered to be very important.

The experimental results mentioned above provide conclusive proof that the carboxyl group (-COOH) is the essential ionizable group of honeybee trehalase in the alkaline region.

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