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COMPARISON OF FUNGAL GLUCOSE OXIDASES

CHEMICAL, PHYSICOCHEMICAL AND IMMUNOLOGICAL STUDIES

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Summary

Glucose oxidases (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) from two fungal genera (*Aspergillus* and *Penicillium*) were studied chemically, physicochemically and immunologically to elucidate the similarities and dissimilarities between these enzymes. Investigation of circular dichroism spectra revealed that these enzyme proteins possess essentially identical conformations. However, differences found in thermal inactivation parameters, catalytic parameters and quantitative immunological reactivities indicate that these enzymes must have some minor but distinct variations in their structures. Interestingly, it was observed that the *Penicillium* enzyme cross-reacted with the antiserum against the *Aspergillus* enzyme with an association constant of two orders of magnitude lower than that of the *Aspergillus* enzyme, and that the precipitin line of the *Penicillium* enzyme fused together with that of the *Aspergillus* enzyme in the immunodouble diffusion test.

These results lead to the conclusion that these enzymes are closely related but not completely identical, and suggest that they might have evolved from a common ancestral precursor.

Introduction

In 1926 Müller [1] reported the presence of glucose oxidase (EC 1.1.3.4) in *Aspergillus niger* and *Penicillium glaucum*. Since then, many investigators have purified similar or identical enzymes to homogeneity from several fungal species [2—7]. It is known that these enzymes possess many properties in common: for example, (1) the molecular weight of the enzyme is around 150 000; (2) each enzyme molecule contains two molecules of FAD; (3) all the enzymes so far tested are known to be glycoproteins; and (4) all enzymes are highly specific to the sugar substrate, and the reaction mechanisms investigated with these enzymes are virtually identical [8—10]. On the other hand, by detailed

examination of the literature, many nonidentical properties between *Aspergillus* enzymes and *Penicillium* enzymes can also be pointed out: (1) some physicochemical characteristics such as absorbance spectra and partial specific volumes are different [3,6]; (2) the carbohydrate contents and the amino acid compositions are somewhat varied [6,7,11]; (3) the semiquinoid FAD can be produced by dithionite titration with *Aspergillus* enzymes, but not with a *Penicillium* enzyme [6,12]; (4) the subunit compositions are reportedly different [7,13,14]; and (5) a *Penicillium* enzyme is known to be excreted into the culture medium [2] while an *Aspergillus* enzyme remains in the mycelia during submerged culture [15]. However, none of these properties by itself can differentiate these enzymes, and more investigation is required in order to draw a conclusion as to whether these enzymes should be regarded as identical or not. In this context, chemical and physicochemical investigations were carried out comparatively with four enzyme samples supplied from different companies. In addition, the immunological cross-reactivities of these enzymes were examined. The present paper describes and discusses the results.

Materials and Methods

Materials

Aspergillus niger glucose oxidases were obtained from three companies: Kyowa Hakko Kogyo, Tokyo; Boehringer, Mannheim; and Sigma, St Louis. *Penicillium amagasakiense* glucose oxidase was a gift from Nagase Sangyo Co., Osaka. Lyophilized guinea-pig serum (complement), rabbit anti-sheep erythrocyte and sensitized sheep erythrocytes were obtained from Nippon Biotest Laboratory, Tokyo. Chemicals used were of reagent grade.

Purification of glucose oxidase

Kyowa's enzyme was purified from the crude sample by the method described elsewhere [6]. Boehringer's enzyme, "degree of purity I", and Sigma's enzyme, "type II", were further purified by using a Biogel P-150 column and a DEAE-cellulose column. Nagase's enzyme, "GOD-100", was purified by the method of Yoshimura and Isemura [13]. All purified samples were shown to be homogeneous by ultracentrifugation, and each sample showed a single band on a disc gel electrophoresis at pH 8.9. The concentrations of the enzymes were conveniently expressed in terms of the bound FAD and were estimated spectrophotometrically using the extinction coefficient of 10.8 mM^{-1} cm^{-1} at 452 nm for the *Aspergillus* enzymes and at 460 nm for the *Penicillium* enzyme [6].

Carbohydrate analyses.

Total carbohydrate contents were estimated by the phenol/sulfuric acid method [16] with mannose as the standard. Quantitative and qualitative analyses of neutral sugar components were carried out with a Shimadzu gas-chromatograph, GC-4BM. Amino sugar components were determined by the Elson-Morgan method [17] and by use of an amino acid analyzer, Hitachi 034.

Spectrophotometric measurements

Spectrophotometric measurements were made with a Hitachi double-beam spectrophotometer, Model 124, at room temperature.

Circular dichroism spectra

Circular dichoism spectra were measured at room temperature with a Jasco Spectropolarimeter, J-20.

Ultracentrifugation

The ultracentrifuge analyses were done by the use of a Beckman Model E ultracentrifuge at 20° C.

Enzyme assay

The catalytic activities of the enzymes were measured by oxygen uptake at 25° C in a 0.05 M acetate buffer, pH 5.5, as described in a previous report [6].

Thermal inactivation kinetics

A solution of glucose oxidase was incubated in a water bath at a desired temperature, and an aliquot was taken out at intervals for the test of the residual activity. The assay was performed in the presence of 57 mM D-glucose. The inactivation was known to follow first-order kinetics during more than 70% of the total process. The first-order rate constant, k_d, was measured at various temperatures and was used to determine the activation energy, $E\bigstar$, of the thermal inactivation process by the use of the Arrhenius plot.

Immunological experiments

Antisera were prepared by immunization of rabbits with the purified glucose oxidases from *Aspergillus niger* (Kyowa) and *Penicillium amagasakiense* (Nagase). An emulsion of the enzyme with Freund's complete adjuvant was injected subcutaneously in the foodpads at a dosage of 3 mg protein per kg weight. Once every three weeks the antigen was injected in the same manner but without the adjuvant. After 2 months, the antiserum was prepared from the rabbit. Preimmune serum was prepared from a rabbit before injection of the antigen enzymes. The sera were stored at −15° C until use. Immunodouble diffusion experiments were carried out on agar plates as described by Ouchterlony [18]. Quantitative precipitin reaction and the complement fixation tests were performed essentially after McDuffie and Kabat [19] and Wasserman and Levine [20], respectively.

Results

Carbohydrate contents

The total carbohydrate contents and the amounts of the component sugars estimated with the present preparations are listed in Table I. Among the *Aspergillus* enzymes, Sigma's enzyme showed the highest total carbohydrate content. The total carbohydrate content of Kyowa's enzyme was somewhat lower, and that of the *Penicillium* enzyme (Nagase) was a little higher than the respective values reported previously [6]. It is interesting to note that all enzyme samples were found to contain mannose as the main carbohydrate component, and significant amounts of hexosamine, which was identified as glucosamine by the use of the amino acid analyzer. This is indicative of the occurrence of a common glycopeptide structure in these enzymes, and further studies are required.

TABLE I

CARBOHYDRATE CONTENTS OF GLUCOSE OXIDASES

Glucose oxidase	Total carbohydrate content (%)	No. of residues per mol of bound FAD				
		Man	Gal	Glc	Hexosamine *	(GlcN) **
Aspergillus niger						
Kyowa	13.0	54	4	1	10	(7.4)
Boehringer	10.7	43	1.5	trace	9	(7.6)
Sigma	16.5	77	2	trace	9	(7.1)
Penicillium amagasakiense						
Nagase	13.5	53	7	2.5	14	(6.6)

* Determined by Elson-Morgan method.

** Determined by an amino acid analyser.

Circular dichroism spectra

As shown in Fig. 1, all the enzymes showed almost the same profile of the circular dichroism spectrum, indicating that there is no significant difference in the protein structures of the *Aspergillus* enzymes and the *Penicillium enzyme*. In agreement with our previous paper [21], glucose oxidases are known to contain relatively small amounts of ordered conformations, such as α-helix and β-pleated sheet structures. Assuming that these circular dichroism patterns are due to α-helix conformation, the helical contents of these enzymes are calculated to be approximately 15% [22,23].

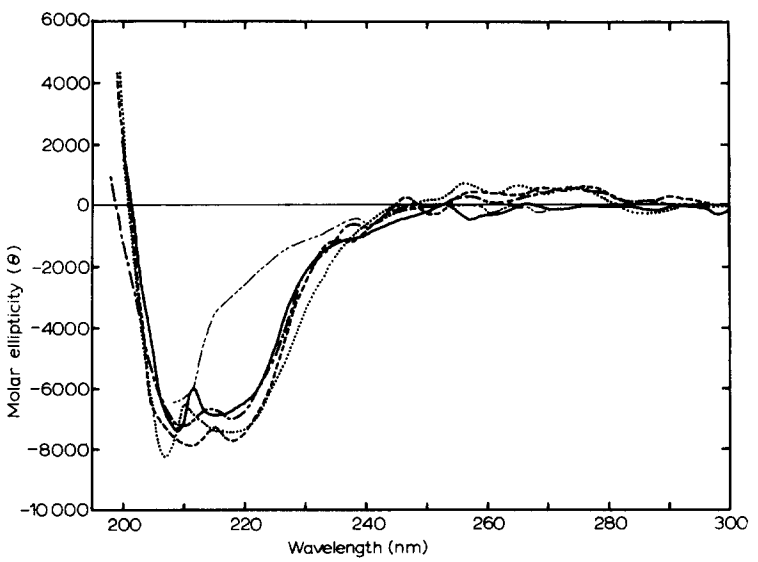

Fig. 1. Circular dichroism spectra of glucose oxidases. The spectra were measured with a 1-mm cell at room temperature. In the calculation of molar ellipticity, [Θ], the mean residue weight of the amino acids was taken as 109 on the basis of the amino acid compositions [6]. (———), Kyowa's enzyme; (- - - - -), Boehringer's enzyme; (- · - · - ·), Sigma's enzyme; (. . . . .), Nagase's enzyme; and (- · · -), 6 M urea-denaturated Kyowa's enzyme.

Thermal inactivation kinetics

In order to get further insight into the protein structures of the enzymes, thermal inactivation processes were investigated. The first-order rate constants, k_d, for the inactivation processes obtained at various temperatures are given in Fig. 2 in the form of the Arrhenius plot. All the enzymes from the *Aspergillus* species showed much the same behavior against heating (Curves a, b and c), but the *Penicillium* enzyme behaved differently (Curve d). Table II presents the values of the activation parameters calculated from the experimental data in Fig. 2. The value of the activation enthalpy for the *Penicillium* enzyme was distinct from those for the *Aspergillus* enzymes. To be noted here is the finding that the free energy of activation was nearly constant in all cases. As a result, the value of the entropy of activation for the *Penicillium* enzyme significantly exceeded those of the *Aspergillus* enzymes.

Overall reaction kinetics

The overall catalytic reactions were investigated in the presence of varying concentrations of glucose, and the kinetic parameters were estimated by the Lineweaver-Burk plots. As shown in Table III, the turnover number, *V/e*, of the *Penicillium* enzyme was approximately twice as large as those of the *Aspergillus* enzymes, and the value of the Michaelis constant for glucose was considerably lower for the *Penicillium* enzyme as compared with those for the *Aspergillus* enzymes. These results indicate that the *Penicillium* enzyme is highly distinguished catalytically from the *Aspergillus* enzymes, while the latter are almost identical with each other irrespective of their manufacturers.

Immunodouble diffusion studies

With each enzyme from *Aspergillus niger*, one sharp, mutually-fused precip-

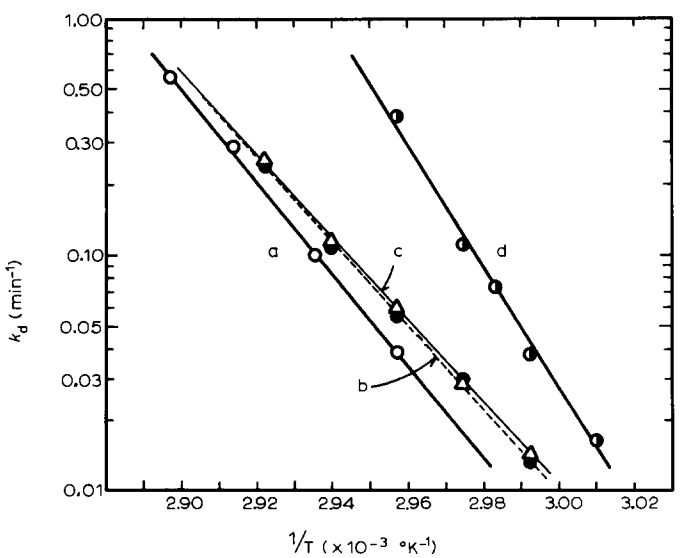

Fig. 2. Arrhenius plots ror thermal inactivation of glucose oxidases. (a) Kyowa's enzyme (b) Boehringer's enzyme, (c) Sigma's enzyme and (d) Nagase's enzyme.

TABLE II

ACTIVATION PARAMETERS FOR THERMAL INACTIVATION OF GLUCOSE OXIDASES

Activation parameters were calculated by the following equations: $\Delta H^{\ddagger} = E - RT$, $\Delta F^{\ddagger} = -RT \cdot \ln[(h/kT)k_d]$, and $\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta F^{\ddagger})/T$, where $\Delta H^{\ddagger}$, $\Delta F^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy, free energy and entropy of activation; R, h and k are the gas constant, Planck's constant and Boltzmann's constant, respectively. Values listed here are the average of four to five calculations on the basis of the experimental data in Fig. 2.

Glucose oxidase	$\Delta H^{\neq}$ (kcal/mol)	$\Delta F^{\neq}$ (kcal/mol)	$\Delta S^{\neq}$ (cal/°K per mol)
Aspergillus niger			
Kyowa	87.3	24.1	184.8
Boehringer	81.8	24.6	169.2
Sigma	80.3	24.5	164.9
Penicillium amagasakiense			
Nagase	118.8	24.2	282.1

itin line was obtained with the antiserum against the *Aspergillus* enzyme (Kyowa), but the cross-reactivity of the *Penicillium* enzyme was not apparent (Fig. 3a). With about a 10-fold increase in the amount of the *Penicillium* enzyme, a definite precipitin line became observable (Fig. 3b). It should be noted that the precipitin line by the *Penicillium* enzyme fused together with the line by the *Aspergillus* enzyme. In Fig. 3c, the precipitin tests of these enzymes with the antiserum against the *Penicillium* enzyme are shown. The *Penicillium* enzyme showed a sharp single precipitin line, while those from the *Aspergillus* species all failed to cross-react with the anti-*Penicillium* enzyme. In this case, no precipitin line was observed, even with a 100-fold increased amount of the *Aspergillus* enzyme (Kyowa). To confirm these findings, quantitative precipitation and complement fixation tests were carried out.

Quantitative immunoprecipitation

Quantitative immunoprecipitation curves obtained with the anti-*Aspergillus* enzyme (Kyowa) and the anti-*Penicillium* enzyme are shown in Fig. 4. The cross-reactivities of the *Aspergillus* enzymes with the anti-*Aspergillus* enzyme

TABLE III

APPARENT KINETIC PARAMETERS OF GLUCOSE OXIDASES

Activity was measured at an oxygen concentration of 260 μM.

Glucose oxidase	V/e * (min^{-1})	K'_m for glucose (mM)
Aspergillus niger		
Kyowa	10 100	26
Boehringer	14 800	30
Sigma	9 590	30
Penicillium amagasakiense		
Nagase	24 500	11

* e: total enzyme concentration in terms of the bound FAD.

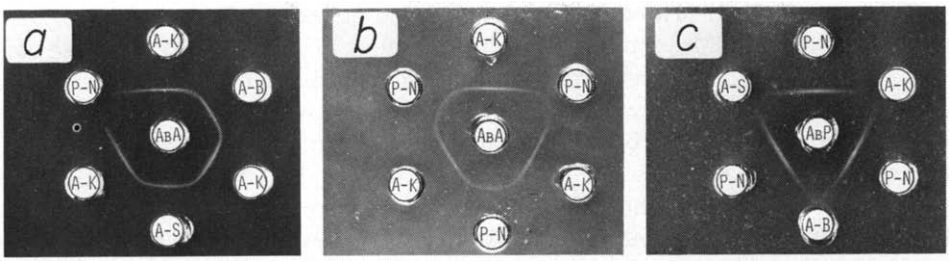

Fig. 3. Agar immunodouble diffusion experiments with the anti-*Aspergillus* enzyme (Kyowa) and anti-*Penicillium* enzyme (Nagase). Abbreviations used in this figure are: *ABA*, antiserum against the *Aspergillus* enzyme; ABP, antiserum against the *Penicillium* enzyme; A-K, Kyowa's enzyme; A-B, Boehringer's enzyme; A-S, Sigma's enzyme; and P-N, Nagase's enzyme. (a) Cross-reactivities of glucose oxidase with the anti-*Aspergillus* enzyme. *ABA*, 074 mg; A-K, 7.3 μg; A-B, 7.5 μg; A-S, 8.6 μg; and P-N, 7.8 μg. (b) Cross-reactivity of the *Penicillium* enzyme with the anti-*Aspergillus* enzyme at a high protein concentration. *ABA*, 0.37 mg; A-K, 4.3 μg; P-N, 58 μg. (c) Cross-reactivities of glucose oxidases with the anti-*Penicillium* enzyme. *ABP*, 0.7 mg; A-K, 7.3 μg; A-B, 7.5 μg; A-S, 8.6 μg; and P-N, 3.9 μg.

were all quantitatively identical (Curve 1). The immunological association constants estimated by the double reciprocal plots were $3.2 \cdot 10^6$, $3.6 \cdot 10^6$ and $5.0 \cdot 10^6$ M^{-1} for Kyowa's enzyme, Boehringer's enzyme and Sigma's enzyme, respectively. The cross-reactivitiy of the *Penicillium* enzyme with this antiserum was as shown in Curve 2. That the precipitin reaction really resulted from the antigen-antibody interaction between the *Penicillium* enzyme and the anti-*Aspergillus* enzyme is supported by the observation that the *Penicillium* enzyme did not form any appreciable amount of precipitates with the preimmune

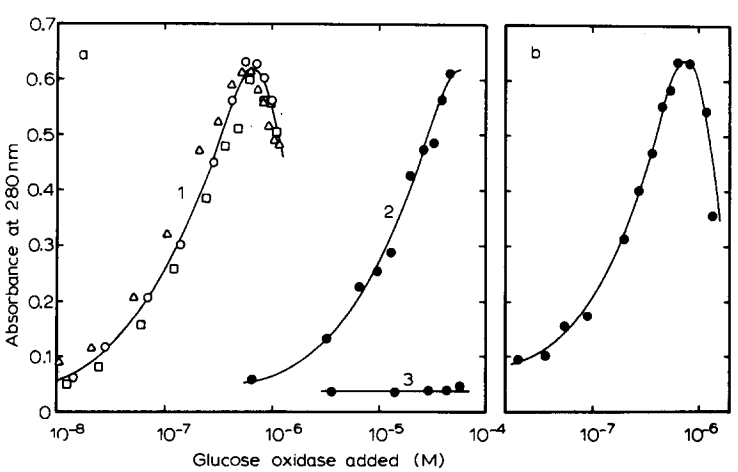

Fig. 4. Quantitative immunoprecipitin reactions of glucose oxidases with the anti-*Aspergillus* enzyme (Kyowa) and the anti-*Penicillium* enzyme (Nagase). Antiserum was incubated with varying amounts of glucose oxidase (antigen) in 0.1 M phosphate buffer, pH 7.0, for 3 h at 37°C in a total volume of 1.1 ml; then the precipitates were collected. After washing with 0.85% NaCl, the precipitates were dissolved in 0.5 ml of 0.1 M NaOH and the absorbance at 280 nm was measured. (a) Precipitin reactions of glucose oxidases with the anti-*Aspergillus* enzyme. Curve 1: reactivities of the *Aspergillus* enzymes; (○) Kyowa's enzyme, (□) Boehringer's enzyme, and (△) Sigma's enzyme. Curve 2: reactivity of the *Penicillium* enzyme with the anti-*Aspergillus* enzyme. Curve 3: reactivity of the *Penicillium* enzyme with preimmune rabbit serum. Anti-*Aspergillus* enzyme, 7.5 mg. Premmune serum, 7.5 mg. (b) Precipitin reaction of the *Penicillium* enzyme with the anti-*Pencillium* enzyme. Anti-*Penicillium* enzyme, 7 mg.

rabbit serum (Curve 3). The association constant estimated for the *Penicillium* enzyme was $5.8 \cdot 10^4$ M^{-1}, about two orders of magnitude lower than those for the *Aspergillus* enzymes. These results are in good agreement with the observations of the Ouchterlony tests shown in Fig. 3. The precipitin reaction of the *Penicillium* enzyme with its own antiserum is shown in Fig. 4b. The association constant estimated from this figure was $2.0 \cdot 10^6$ M^{-1}, which was approximately identical with those of the *Aspergillus* enzymes for the anti-*Aspergillus* enzyme. No significant precipitate was observed for any of the *Aspergillus* enenzymes with the anti-*Penicillium* enzyme.

Complement fixation tests

The antiserum directed against the *Aspergillus* enzyme was tested for complement fixation with both the *Aspergillus* and the *Penicillium* enzymes. The results are shown in Fig. 5. With 7.4 μg of the antiserum in 3.5 ml, the optimum regions of fixation were around $5 \cdot 10^{-13}$ M in the case of the *Aspergillus* enzyme and $5 \cdot 10^{-10}$ M in the case of the *Penicillium* enzyme, respectively. These results are again consistent with the results of other immunological experiments, as already seen in Fig. 3 and 4.

Effects of antisera on catalytic activity

No significant inhibitory effect of these antisera was observed with any of these glucose oxidases, in accord with other reports [24,25]. It is therefore

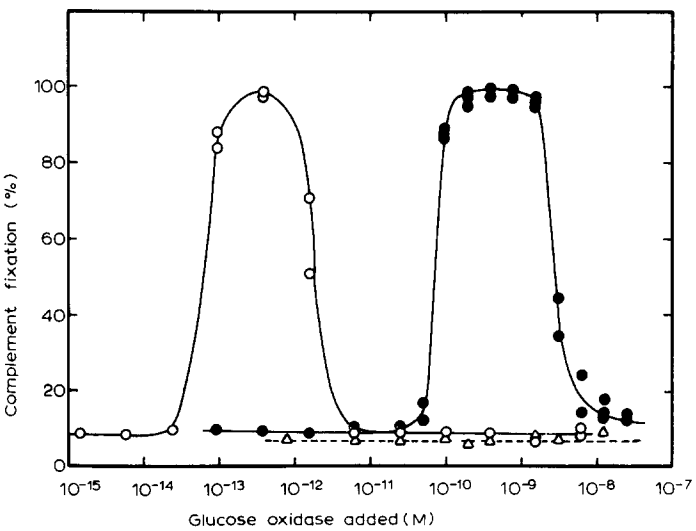

Fig. 5. Quantitative complement fixation of glucose oxidases with the anti-*Aspergillus* enzyme (Kyowa). The reaction mixture, containing 1.5 ml of diluent (isotonic Veronal buffer, pH 7.5, with 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 0.15 M NaCl and 0.1% bovine serum albumin), 0.5 ml of complement solution (a 250-fold diluted lyophilized guinea pig serum), 0.5 ml of glucose oxidase solution (antigen), and 0.5 ml of the anti-*Aspergillus* enzyme, was incubated at 4°C for 48 h, then 0.5 ml of the sensitized erythrocyte suspension was added. After allowing hemolysis at 37°C for 1 h, hemoglobin was analyzed spectrophotometrically at 413 nm. Solutions with an excess and without complement were used as controls for 100% and 0% of the fixation, respectively. (○) *Aspergillus* enzyme (Kyowa); (●) *Penicillium* enzyme in the presence of the anti-*Aspergillus* enzyme; and (△) *Penicillium* enzyme in the absence of the antiserum. Anti-*Aspergillus* enzyme, 7.4 μg.

conceivable that the antigenic sites are far removed from the catalytic sites on these proteins, or the substrate molecules (glucose and molecular oxygen) are too small to be hindered by the antibody molecules.

Discussion

Glucose oxidases of fungal origins are at present commercially available from several companies. Most of these enzymes are from *Aspergillus niger* and a few from *Penicillium* species. Although many papers, which described the properties of glucose oxidases, have appeared, few of them presented comparative studies on these enzymes. One of the problems remaining to be studied involves the comparison of the *Aspergillus* enzymes with the *Penicillium* enzymes; and another involves the comparison between the enzymes of the same fungal genus but from different manufacturers.

As to the *Aspergillus* enzymes from three companies, it was shown in the present study that they share many properties in common, such as kinetic parameters, thermal inactivation parameters and immunological reactivities. As a whole, it is reasonable to conclude that these enzymes are identical. The small differences observed in the carbohydrate contents may probably be due to the differences of culture conditions or to the methods of purification adopted by the manufacturers, and hence, these variations may not be taken as criteria for differentiation of these enzymes.

The *Penicillium* enzyme, on the other hand, was shown to possess apparently different characteristics from the *Aspergillus* enzymes. Accordingly, these enzymes can be regarded as, at least in a quantitative sense, nonidentical. However, more detailed discussion will be required before we may draw a conclusion as to the relationship between these enzymes from the two different fungal genera.

The protein structures

As already noted, the secondary protein structures of the *Aspergillus* and the *Penicillium* enzymes are roughly identical.

The amino acid compositions of these enzymes have been reported by three independent groups [6,7,11]. By examining these reports, the enzymes are seen to have roughly identical amino acid compositions: they contain large amounts of acidic amino acid residues and relatively small amounts of basic residues. There are, however, minute but distinct variations of the compositions; the *Penicillium* enzymes contain larger amounts of lysyl and phenylalanyl residues and smaller amounts of histidyl and tyrosyl residues than the *Aspergillus* enzymes.

Activation parameters for thermal inactivation

The activation enthalpy and entropy of the *Penicillium* enzyme exceeded those of the *Aspergillus* enzymes to a significant degree. This does not mean, however, that the *Penicillium* enzyme is thermally more stable than the *Aspergillus* enzymes, since it is evident from Fig. 2 that the inactivation of the *Penicillium* enzyme took place at a lower temperature range than those of the *Asper-*

gillus enzymes. Rather, the higher values should be considered as the indication of a higher cooperativity of the protein molecule in thermal inactivation process. The differences in the activation parameters can therefore be regarded as evidence for a difference in the structures between the *Penicillium* enzyme and the *Aspergillus* enzymes.

Immunological reactivities

When evidence for a homologous relationship between protein structures is being sought, it is often advantageous to examine immunological cross-reactivities between the proteins in question. In accordance with other reports [24, 25], glucose oxidases, both the *Aspergillus* and the *Penicillium* enzymes, could produce their respective antibodies in rabbit sera. As shown in Figs. 3 and 4, the *Aspergillus* enzymes failed to cross-react with the anti-*Penicillium* enzyme. It is thus evident that the *Aspergillus* enzymes are immunologically distinct from the *Penicillium* enzyme. Important findings are that the *Penicillium* enzyme cross-reacted with the anti-*Aspergillus* enzyme with an association constant of two orders of magnitude lower than those of the *Aspergillus* enzymes, and that the precipitin line of the *Penicillium* enzyme fused together with that of the *Aspergillus* enzyme on the agar plate. There are several possible explanations for these results. (1) The *Penicillium* enzyme possesses a similar but slightly modified antigenic determinant to that of the *Aspergillus* enzyme, so that the anti-*Aspergillus* enzyme can bind to the *Penicillium* enzyme weakly, but not vice versa. (2) The *Penicillium* enzyme has an antigenic determinant quite identical to that of the *Aspergillus* enzyme, but some factors such as steric hindrance or conformational difference in the environment may interfere with the interaction between the *Penicillium* enzyme and the anti-*Aspergillus* enzyme. On the other hand, the determinant for the anti-*Penicillium* enzyme is not shared by the *Aspergillus* enzyme. (3) The *Penicillium* enzyme is a mixture of protein molecules with and without the antigenic determinant for the anti-*Aspergillus* enzyme, while the antigenic determinant specific to the anti-*Penicillium* enzyme is not shared by the *Aspergillus* enzyme.

The third possibility will be less probable, since the enzyme sample used was highly purified, and it is difficult to think that a micro-heterogeneous component of the *Penicillium* enzyme happens to have an antigenicity identical with that of the *Aspergillus* enzyme. The other two possibilities might equally be probable when judged by the chemical and physico-chemical results presented in this paper. However, if the second possibility is assumed, the rabbit immunized by the *Penicillium* enzyme should also produce the anti-*Aspergillus* enzyme (i.e., a common antibody). This is because the primary structure of the antigen is the most important, rather than the three-dimensional structure, when an antigenic determinant is being recognized. Accordingly, the first possibility may be the most probable in explaining the observed immunological differences.

It has been well demonstrated by the experiments with synthetic antigens [26] or with a series of lysozymes [27—29] and cytochrome *c* [30—33] that the antigen-antibody interaction is, in general, such a sensitive reaction that it can even recognize minor alteration(s) in the primary and/or tertiary structure(s) of proteins. Then the observed lack of cross-reactivity of the anti-*Peni-*

cillium enzyme with the *Aspergillus* enzymes does not necessarily exclude the possibility that their protein structures are highly homologous. Rather than this, the cross-reactivity observed between the *Penicillium* enzyme and the anti-*Aspergillus* enzyme should be emphasized.

Conclusion

Taking into account all these experimental results, it is relevant to conclude that glucose oxidases from both *Aspergillus* and *Penicillium* species share much the same structure in common, and at the same time, they also contain some portions not commonly shared and/or partly modified.

It is tempting to assume that these enzymes may have evolved from a common ancestral precursor. Hence, they still possess many identical or similar properties, but they also have several altered characteristics which have been acquired during evolution. This idea will be interesting if one thinks of the classification of fungal species. The genera *Aspergillus* and *Penicillium* are believed to be closely related by the classification criteria currently adopted. The results shown in the present paper may give some idea as to the way and the method for microbial classification.

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