Biochimica et Biophysica Acta, 657 (1981) 40—51

BBA 69163

MULTIPLE FORMS OF GLUCOSE OXIDASE WITH DIFFERENT CARBOHYDRATE COMPOSITIONS

SUEKO HAYASHI and SATOSHI NAKAMURA

Department of Biophysical Chemistry, Kitasato University School of Medicine, Sagamihara, Kanagawa 228 (Japan)

(Received July 11th, 1980)

Key words: Glucose oxidase; Carbohydrate composition; Multiple forms; (Aspergillus niger)

Summary

A glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) sample purified from *Aspergillus niger* by a previously reported method was subjected to isoelectric focusing and gel electrophoresis, and found to be composed of at least six component enzymes. The isoelectric points of the component enzymes ranged from pH 3.9 to 4.3. Analyses of the enzymes indicated that they all possess an identical protein moiety, since the amino acid compositions, the C-terminal sequences, the catalytic parameters, the quantitative and qualitative immunological properties and the electrophoretic patterns of the peptide fragments, obtained by the CNBr-cleavage, were practically the same. On the other hand, the carbohydrate contents of the isolated component enzymes were found to be different, and these differences were associated in the main with a particular peptide fragment. We suggest that the multiplicity of the enzyme is due to variation in the carbohydrate contents and their structures, rather than in the protein moiety.

Introduction

Several purification procedures and the general properties of fungal glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) have been reported earlier [1—6]. The preparations were regarded as homogeneous by ultracentrifugation and electrophoretic analysis. The enzyme contains 2 mol FAD/mol M_r 153 000 [7]) and 10—16% carbohydrate [4,5,8—10].

In the course of the studies on various glucose oxidase samples, it was noticed that the protein on an isoelectric focusing gel was stained as multiple bands, indicating the microheterogeneity of this enzyme. In order to gain

insight into the cause of the multiplicity, various analyses have been performed on the isolated component enzymes.

Parts of these data have been presented earlier [11].

Materials and Methods

Materials. Acrylamide, *N,N'*-methylene bisacrylamide and DEAE-cellulose were obtained from Seikagaku Kogyo, Tokyo; *N,N,N',N'*-tetramethylethylene diamine, riboflavin and Coomassie brilliant blue R-250 were from Nakarai Chemicals, Tokyo; and ampholytes were from LKB-Produkter, Sweden. Chemicals were of reagent grade.

Crude samples of *Aspergillus niger* glucose oxidase, Lot No. 93 005, were purchased from Kyowa Hakko Kogyo, Tokyo, and purified as described elsewhere [5]. The concentration of the enzyme was determined spectrophotometrically by the use of the extinction coefficient: $\epsilon_{452} = 21.6\ mM^{-1} \cdot cm^{-1}$ [5].

Polyacrylamide gel electrophoresis in cylindrical and slab gels. These were performed according to Davis [12]. The cylindrical were 7.5% acrylamide, run at 3 mA/tube. Slab gel electrophoresis was in the presence of 0.1% sodium dodecyl sulfate (SDS) with a gradient of 10—20% acrylamide, and the current applied was 30 mA/plate (137 × 110 × 2 mm).

Isoelectric focusing in polyacrylamide gel. Isoelectric focusing was carried out as described by Doerr and Chrambach [13] with slight modifications. The polymerization mixture contained the following components (final concentrations): 3.36% acrylamide/0.14% *N,N'*-methylene bisacrylamide/0.13 μM riboflavin/6.6 μM ammonium persulfate/1% carrier ampholytes/12.5% glycerol (v/v). This mixture was deaerated under reduced pressure for 2 min and then 20 μl *N,N,N',N'*-tetramethylethylene diamine/10 ml gel solution was added. 1.8 ml of this solution were pipetted into a glass tube (5 × 100 mm) which was closed off at the bottom with Parafilm. A 1-cm layer of water was carefully placed at the top to ensure a flat surface and to complete polymerization. The solution was allowed to gel at room temperature under white light, in order to accelerate polymerization. After polymerization, the upper gel surface was rinsed with distilled water and dried by blotting the top of the gel tube with adsorbent paper. The enzyme solution was diluted with 25% glycerol and 10—20 μl of this mixture were layered onto the gel surface, followed by 'overlayering' with 100 μl 5% glycerol and 0.2% sulfuric acid up to the top of the gel tube. The best results were obtained when no salt or buffer was present. The gels were immersed in electrolyte solutions: 0.2% sulfuric acid at the anode (upper reservoir) and 0.4% ethylene diamine at the cathode (lower reservoir). Voltage was applied and gradually increased to a maximum value of 200 V, at which it was maintained for 8—9 h.

Isolation of component enzymes. The major component enzymes (I to VI, see Fig. 1B) were prepared from the electrofocused enzyme sample by extracting the sliced gels with 0.05 M acetate buffer, pH 5.4. Each of the extracts of the component enzymes was subjected to a DEAE-cellulose column chromatography after overnight dialysis against the buffer. The eluates were each concentrated by ultrafiltration in a Diafilter, MC-1, with a membrane G-10T, from Bio-Engineering Co., Tokyo.

Measurement of pH of the electrofocusing gel. After electrofocusing, gel was cut into 5-mm thick slices and incubated with 1.5 ml distilled water for at least 6 h. The pH values of the incubation mixtures were measured with a Corning pH-meter, Digital 112, equipped with a microelectrode.

Staining gels for protein. This was carried out by the method of Vesterberg [14] with Coomassie brilliant blue R-250.

Staining gels for carbohydrate. To stain the carbohydrate moiety of glucose oxidase, the periodic acid-Schiff method was used according to Zaccharius et al. [15].

Amino acid analyses. Amino acid analyses were done using a JEOL amino acid analyzer, JLC-6AH, with the samples hydrolyzed by 6 N HCl for 22, 48 and 72 h, or with those hydrolyzed by 4 N methanesulfonic acid for 22, 48 and 72 h according to Simpson et al. [16]. By the latter method, tryptophan and half-cystine residues were determined concomitantly with other amino acid residues by use of a single hydrolysate. The number of the amino acid residues per enzyme molecule were calculated on the molar basis of the enzyme-bound FAD.

Carbohydrate analyses. Total carbohydrate content of the enzyme was estimated by the phenol-sulfuric acid method with D-mannose as the standard [17]. Qualitative and quantitative analyses of neutral sugar components were carried out by the use of a JEOL gas-chromatograph, JBC-1100, after complete *O*-methylation [18]. Amino sugar components were determined by the use of the amino acid analyzer. Sialic acid analysis was performed according to Aminoff [19].

Analysis of the N-terminal amino acid residue. The dansylation [20] and leucine aminopeptidase methods [21] were applied to determine the N-terminal amino acid residue.

Analysis of the C-terminal amino acid sequence. The C-terminal amino acid sequences of the component enzymes were determined by the use of carboxypeptidases A and B according to Ambler [22].

CNBr-cleavage of component enzymes. Approx. 1 mg glucose oxidase sample was dissolved in 0.1 ml 70% formic acid, containing $1.15 \cdot 10^{-4}$ mol CNBr, to provide a 400-fold molar excess of CNBr over the amount of methionine contained in the protein (about 23 residues per molecule of enzyme, see Table II). The reaction was allowed to proceed for 24 h at 25°C with stirring. The reaction was found to be almost completed in this time period, judging from the results of homoserine analysis. The reaction mixture was then diluted 5-fold with 0.4 ml distilled water and was freeze-dried.

Immunological experiments. Antiserum against the purified glucose oxidase sample was prepared as reported previously [10]. Immuno double-diffusion tests were carried out by the method of Ouchterlony [23]. Quantitative precipitin experiments were done as reported by McDuffie and Kabat [24].

Catalytic activity. The overall catalytic activity of glucose oxidase sample was estimated by oxygen uptake at 25°C in a 0.05 M acetate buffer, pH 5.5, by the use of a Clark oxygen electrode.

Densitometric measurements. A Fuji Riken densitometer, FD-A, was used for scanning the stainings of the electrofocused gels with a filter for 570 nm.

Spectrophotometric measurements. The spectrophotometer used was a

Hitachi double-beam spectrophotometer, Model 124.

Circular dichroism measurements. The circular dichroism (CD) measurements were made with a Union Giken Dichrograph, III-J, at 25°C, which was interfaced to a computer, Union Giken System-77.

Results

Isoelectric focusing in polyacrylamide gel

A glucose oxidase sample purified by the reported method [5] shows a single and symmetric boundary either on ultracentrifugation or on the electrophoresis in a Tiselius-type apparatus. It was further verified that it migrated as a single band on polyacrylamide gel electrophoresis at pH 9.4, judging either from the staining of the protein moiety or from that of the carbohydrate moiety (Fig. 1A). It was noticed, however, that the stained bands were rather broad as compared with those of other standard proteins of the same quantity under identical conditions. This suggested that the glucose oxidase sample might contain several microheterogeneous species. Isoelectric focusing of the enzyme sample was then performed in the polyacrylamide gel, containing 1% carrier ampholytes with a pH gradient of 3.5—5. As shown in Fig. 1B, at least six bands were recognized by the staining for either protein or carbohydrate. The isoelectric points of these components ranged from pH 3.9 to 4.3. It is to be noted that the densitometric tracing for the protein moiety does not quantitatively coincide with that for the carbohydrate moiety (Fig. 2), indicating that the carbo-

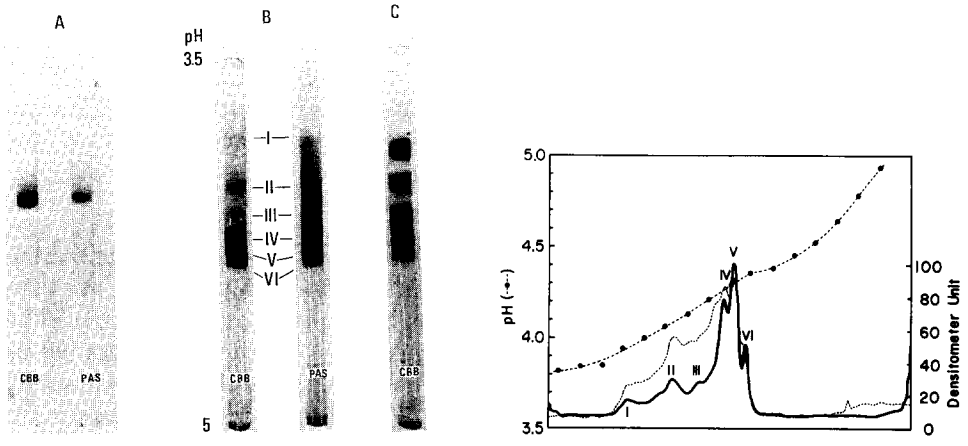


Fig. 1. (A) Polyacrylamide gel electrophoresis of glucose oxidase at pH 9.4 with acrylamide concentration of 7.5%. The gels were stained with Coomassie brilliant blue R-250 (CBB) (left) and periodic acid-Schiff (PAS) (right), respectively. (B) Polyacrylamide gel isoelectric focusing of glucose oxidase in 3.5% acrylamide gel, containing 1% carrier ampholytes of pH 3.5—5. The gels were stained with CBB (left) and PAS (right), respectively. (C) Polyacrylamide gel isoelectric focusing of the mixture of equimolar amounts of the isolated component enzymes. The conditions were the same as in B. The gel was stained with CBB. The amounts of the enzyme applied to the gels were 30 μg (A), 150 μg (B) and 30 μg each of the component enzymes (C), respectively.

Fig. 2. Densitometric scanning traces of the electrofocused gels shown as Fig. 1B. ———, scanning trace for protein; - - - - - -, scanning trace for carbohydrate. The isoelectric points of the component enzymes were: I, 3.88; II, 4.08; III, 4.17; IV, 4.25; V, 4.29 and VI, 4.33.

hydrate contents of the component enzymes are different. The electrofocused gels were sliced and the major component enzymes were extracted. The mixture of the equimolar amounts of the isolated component enzymes were again subjected to gel isoelectric focusing in the same manner as before. The results are shown in Fig. 1C, together with those of the gel of the 'non-separated' enzyme sample. The isoelectric points observed with the isolated component enzymes agreed well with those of corresponding component enzymes in the non-separated enzyme sample. The densitometric pattern of the re-electrofocused gel was, on the other hand, not quantitatively identical with that of the non-separated enzyme sample, being indicative of an equimolar relationship between the isolated component enzymes in accord with the amounts applied on the gel. These results indicate that the multiplicity found by the isoelectric focusing technique is not an artifact, arising from the interaction between the carrier ampholytes and the protein, but the glucose oxidase sample, per se, consists of microheterogeneous species.

Carbohydrate analyses

Since the stainings of the electrofocused enzyme sample indicated that the carbohydrate contents of the component enzymes were different, the quantitative analyses were performed with the isolated component enzyme samples (Table I). The total carbohydrate contents of the non-separated enzyme sample were approx. 9%, which was slightly lower than those reported previously [10]. The carbohydrate contents of the component enzymes were from 8 to 12%, and it was revealed that the components with relatively acidic isoelectric points possessed higher total carbohydrate contents as compared with those with relatively basic isoelectric points. As in the non-separated enzyme sample [10], mannose is the most abundant component sugar in each of the isolated component enzymes. The contents of glucosamine were almost constant with all the component enzymes while those of neutral sugar components, especially mannose, were significantly varied. No sialic acid was detected in any of the enzyme samples.

Amino acid compositions

The isolated component enzyme samples were subjected to amino acid analyses (Table II). Since the molecular size of the enzyme is rather large, we cannot discuss the small differences in the figures of the amino acid residues. The amino acid compositions of the enzyme samples should, thus, be regarded as identical within the errors of analysis. It is then conceivable that the isoelectric difference in the component enzymes may not be due to the differing amino acid compositions.

Analyses of the N-terminal amino acid residue

The N-terminal amino acid residue of the enyzme samples could not be detected by the dansylation method or leucine-aminopeptidase method. It appears highly likely that the N-terminal residue of the enzyme is masked and not accessible to these reagents.

Analyses of the C-terminal amino acid sequence

The C-terminal amino acid sequences of the non-separated and the isolated component enzyme samples were determined by the use of carboxypeptidase A

TABLE I

CARBOHYDRATE COMPOSITIONS OF THE COMPONENT ENZYMES

	Component enzyme					
	I	II	III	IV	V	VI
Isoelectric point	3.88	4.08	4.17	4.25	4.29	4.33
Total carbohydrate content (%)	11.8 ± 0.87	10.24 ± 0.34	9.99 ± 0.13	8.95 ± 0.47	8.46 ± 0.39	7.94 ± 0.34
No. of residue/mol enzyme *						
Mannose	105.6 ± 3.0	84.5 ± 3.7	83.5 ± 1.3	66.8 ± 2.9	62.2 ± 5.7	61.6 ± 4.7
Galactose	11.6 ± 0.8	10.1 ± 1.4	7.0 ± 0.3	4.3 ± 1.5	4.6 ± 0.5	4.7 ± 0.4
Glucose	trace	0.4	—	0.4	0.7	—
Glucosamine	17.5 ± 1.1	17.3 ± 0.9	16.7 ± 0.8	16.8 ± 0.8	16.7 ± 1.1	16.2 ± 1.2

* The figures are the averages of five samples of different preparations.

TABLE II

AMINO ACID COMPOSITIONS OF THE COMPONENT ENZYMES

The numbers of the amino acid residues were calculated on the basis of the amount of enzyme-bound FAD. The figures are the averages of three to five samples of different preparations. All values are in residues/mol.

Amino acid residue	Component enzymes					
	I	II	III	IV	V	VI
Lys	35.9 ± 0.5	35.8 ± 0.9	35.6 ± 0.5	34.8 ± 0.4	35.0 ± 0.3	35.3 ± 0.9
His	31.3 ± 0.2	31.6 ± 1.1	29.6 ± 1.1	32.8 ± 0.3	32.2 ± 0.7	33.1 ± 0.6
Arg	47.5 ± 2.8	50.0 ± 1.2	47.8 ± 2.5	51.9 ± 1.0	51.0 ± 1.0	50.5 ± 0.8
Asx	149.5 ± 3.3	152.8 ± 7.0	150.4 ± 3.9	150.5 ± 3.5	151.9 ± 4.6	149.9 ± 2.4
Thr	84.0 ± 6.0	85.2 ± 2.0	85.5 ± 5.7	87.8 ± 2.3	86.6 ± 1.4	88.5 ± 2.1
Ser	78.5 ± 2.3	75.1 ± 1.9	80.3 ± 2.4	76.4 ± 0.9	76.7 ± 0.8	75.8 ± 0.6
Glx	108.0 ± 3.8	110.4 ± 2.4	114.8 ± 0.9	113.8 ± 1.4	112.4 ± 1.0	111.8 ± 0.9
Pro	57.9 ± 4.7	54.7 ± 1.0	58.2 ± 2.5	55.1 ± 6.2	57.9 ± 4.0	57.6 ± 2.8
Gly	118.5 ± 4.9	127.0 ± 5.3	121.8 ± 6.6	124.2 ± 0.8	124.7 ± 3.1	124.0 ± 1.3
Ala	134.2 ± 0.3	134.9 ± 6.1	134.6 ± 6.1	134.6 ± 3.2	131.6 ± 4.5	133.8 ± 1.7
Cys *	4.6	6.0	5.3	5.0	5.0	5.9
Val	92.5 ± 0.5	87.0 ± 2.8	89.9 ± 2.4	88.7 ± 4.1	86.3 ± 2.3	87.8 ± 2.5
Met	26.0 ± 3.5	22.3 ± 1.4	25.1 ± 2.5	23.0 ± 0.7	23.3 ± 1.0	22.6 ± 0.9
Ile	52.1 ± 3.4	54.1 ± 2.5	50.2 ± 0.7	53.1 ± 0.1	50.3 ± 2.1	53.7 ± 1.0
Leu	103.0 ± 2.7	105.8 ± 4.4	104.6 ± 0.5	105.0 ± 3.2	104.6 ± 3.3	104.9 ± 4.2
Tyr	53.0 ± 1.9	53.8 ± 0.8	51.1 ± 6.3	53.0 ± 0.1	52.7 ± 1.8	53.6 ± 2.2
Phe	37.0 ± 0.9	36.4 ± 0.5	32.6 ± 0.6	35.3 ± 0.6	35.5 ± 1.2	34.9 ± 1.3
Trp *	15.8	16.0	16.1	15.7	17.7	15.5

* These are the averages of two samples analyzed by the method of Simpson et al. [16].

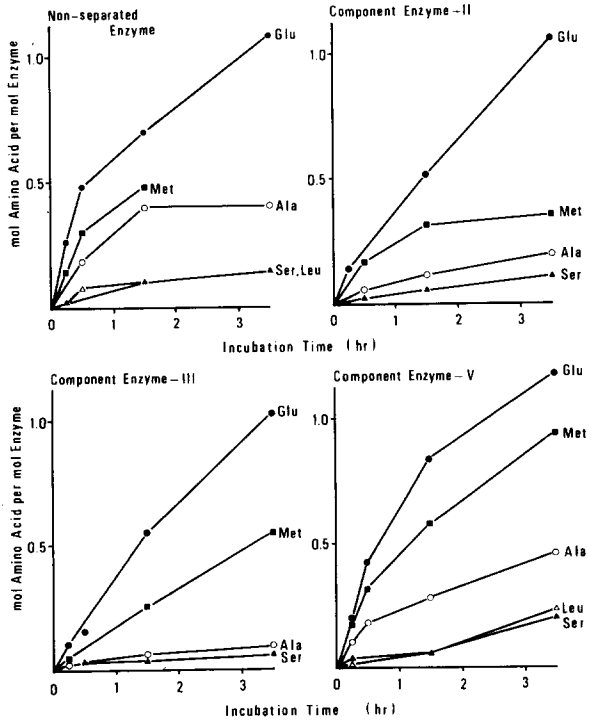


Fig. 3. Rates of release of amino acids from the non-separated and major component enzymes by the action of carboxypeptidase A. The substrate protein (glucose oxidase) concentration was 1% in Tris-HCl buffer containing 3 mM difluorophosphate, pH 8.0. The reaction was initiated by addition of carboxypeptidase A (enzyme/substrate, 1 : 100) at 25°C. Aliquots were withdrawn from the reaction mixture and the reaction was stopped by addition of 0.01 M HCl, lowering the pH to 4. The samples were subjected to quantitative amino acid analyses.

(Fig. 3). The same C-terminal sequence, Ala-Met-Glu-COOH, is shared with all the component enzymes.

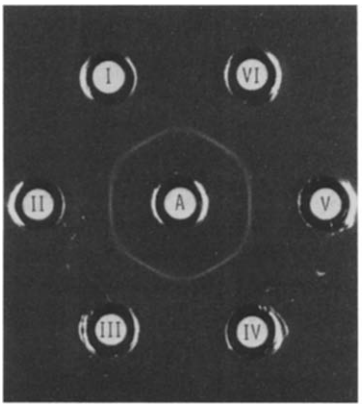
These results together with those of the amino acid compositions and N-terminal amino acid analyses strongly suggest that the primary structures of the component enzymes are all identical.

Immunological properties

The isolated component enzymes were examined by the immuno double-diffusion test against the antiserum to the non-separated enzyme. As Fig. 4 shows, the antiserum cross-reacted with each of the component enzymes and the precipitin lines fused well with each other. The results indicate that the antigenic determinant is commonly shared by these component enzymes.

Quantitative immunoprecipitin test against the antiserum was also performed with the major component enzymes (II, IV, V and VI). It is evident from Fig. 5 that the cross-reactivity of each component enzyme is quantitatively identical. The immunological association constants estimated from the results by the use of the double-reciprocal plots were $5.3 \cdot 10^6$, $6.7 \cdot 10^6$, $7.4 \cdot 10^6$ and $4.4 \cdot 10^6$ M^{-1} for the component enzymes II, IV, V and VI, respectively; and that of the non-separated enzyme was $6.6 \cdot 10^6$ M^{-1}. It is therefore concluded that the immunological properties of the component enzymes are all substantially identical.

This supports the view that the protein moieties of the component enzymes



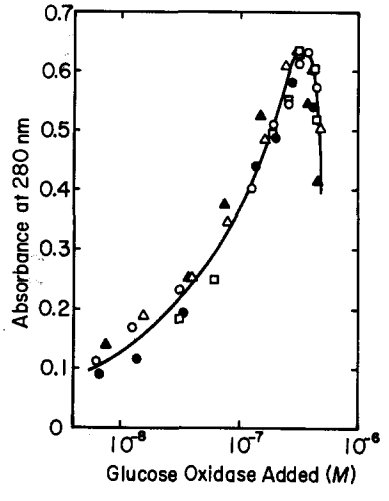


Fig. 4. Agar immuno double-diffusion experiments of the component enzymes with the antiserum against the non-separated enzyme sample. A, I, II, III, IV, V, and VI represent the antiserum, component enzymes I, II, III, IV, V, and VI, respectively. Antiserum, 0.38 mg; each component enzyme, 43 μg.

Fig. 5. Quantitative immunoprecipitin reactions of the non-separated and major component enzymes with the antiserum against the non-separated enzyme. The antiserum was incubated with varying amounts of the component enzymes 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. Reactivities of the non-separated enzyme (●) component enzymes II (▲), IV (△), V (□) and VI (○), respectively. 7.5 mg of the antiserum were added.

TABLE III

APPARENT KINETIC PARAMETERS OF THE COMPONENT ENZYMES

Activity was measured at an oxygen concentration of 260 μM.

	K_m for glucose (mM)	V/e_0 * (min^{-1})
Non-separated enzyme	26	20 200
Component enzyme		
I	26	21 000
II	26	21 000
III	25	19 400
IV	27	20 600
V	27	18 500
VI	25	18 500

* e_0, total enzyme concentration.

are identical, since the antigenic site of the present enzyme is considered to be located on the protein moiety rather than on the carbohydrate moiety [7].

Catalytic properties

The overall reaction catalyzed by the component enzymes was investigated

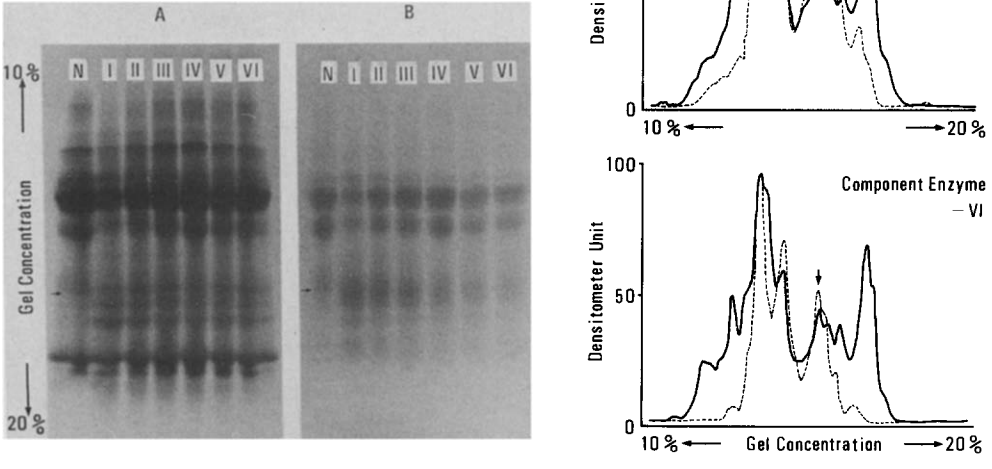


Fig. 6. SDS-polyacrylamide slab gel electrophoresis of the CNBr-cleaved enzyme samples with 10—20% gradient of gel concentration. N: non-separated enzyme; I to VI: component enzymes I to VI, respectively. Each of the CNBr-cleaved samples was dissolved in 1% SDS solution containing 50% glycerol, and an equimolar amount (150 μg each) of the sample was applied to the gel. The gels were stained for protein (A) and for carbohydrate (B), respectively. The other conditions were the same as in Fig. 1.

Fig. 7. Typical examples of densitometric tracings of the CNBr-cleaved glucose oxidase samples (component enzymes I and VI in Fig. 6). Stainings for protein (———) and for carbohydrate (- - - - -), respectively.

in the presence of varying concentrations of glucose, and the apparent catalytic parameters were estimated by the Lineweaver-Burk plots (Table III). No significant difference was detected in the Michaelis constant for the substrate as well as in the turnover number, V/e_0; i.e., all the component enzymes possess essentially identical catalytic properties.

Cleavage of the enzymes by CNBr

The non-separated enzyme and the isolated components enzymes were subjected to CNBr treatment, and the cleaved samples were applied to slab gels in the presence of 0.1% SDS, with a 10—20% acrylamide gradient (Fig. 6). Some 10 peptides were identified on the gel in accordance with the number of methionine residue per subunit of the enzyme (Table II) [25]. Important findings were that the staining patterns of the peptides were practically identical with all the samples tested, indicating that the primary structures are identical, while those of the carbohydrate moieties were quantitatively different. Especially, the peptide indicated by an arrow was found to contain different amounts of carbohydrate depending on the component enzymes. These facts are more clearly demonstrated by the densitometric tracings (Fig. 7), where those of the component enzyme I, the most acidic component with the highest carbohydrate content, and VI, the most basic component with the lowest carbohydrate content, are exemplified. These figures evidently show that the carbohydrate contents of some peptides located in the central region of the gel are dramatically varied depending on the component enzymes.

Discussion

The present investigation has demonstrated by the gel isoelectric focusing technique that the glucose oxidase sample, which was purified from *A. niger* according to practically the same method as currently used in various laboratories, consists of at least six microheterogeneous components. This may be the first report concerning the multiplicity of the present enzyme.

The possibility that the observed heterogeneity is an artifact, arising from the interactions of various degrees between the enzyme and the ampholytes, can be eliminated by the fact that each of the isolated six component enzymes possessed the same isoelectric points as in the non-separated enzyme sample. In addition, the intensities of the stained bands on the gel were not like those of the original sample, apparently showing an equimolar relationship in accordance with the amount of the component enzyme used in the re-electrofocusing. Otherwise, the mixed component enzymes may again be separated to yield an identical densitometric pattern on the second electrofocusing, with that on the first. If bound ampholytes are present, the hydrolysates of the component enzymes will give a ninhydrin-positive peak at a position between those of proline and glycine, when applied to an amino acid analyzer [26]. Since no such peak was observed with the hydrolysates, there cannot be any significant interaction between the ampholytes and the protein. In supporting this, Hayes and Wellner [26] demonstrated by using tritiated ampholytes that little interaction was present between L-amino acid oxidase and the carrier ampholytes in the isoelectric focusing processes. Another possible argument will be that the

multiple species in the glucose oxidase sample may be formed through the action of contaminating glycosidases and/or proteases in the course of the preparation. This is not considered as likely, however, since it is known that glucose oxidase is rather resistant to exogenous glycosidases [7] or proteolytic enzymes [8]. Furthermore, the fact that the amino acid compositions of the isolated component enzymes are essentially identical (Table II), also excludes the possibility of proteolytic attack.

It is therefore possible to conclude that the glucose oxidase sample originally consists of microheterogeneous species.

The microheterogeneity of a glycoprotein, in general, can be ascribed to the difference either in the protein moiety or in the carbohydrate moiety. An example for the former case is L-amino acid oxidase purified from snake venom, which was well investigated by Hayes and Wellner [26]. This enzyme can be separated into at least 18 components by isoelectric focusing techniques, and the multiplicity has been attributed to the variance of their amino acid compositions. Typical examples for the latter case are bovine and porcine pancreatic ribonuclease studies by Plummer and Hirs [27,82] and Reinhold et al. [29], respectively. The multiplicity of the bovine enzyme is due to the presence and absence of a single oligosaccharide moiety; and that of the porcine enzyme is to marked variations in the carbohydrate content.

In the case of glucose oxidase presently being investigated, the former possibility is less probable, since no significant difference was observed with isolated component enzymes in: (1) the amino acid compositions, (2) the C-terminal sequence, (3) the gel electrophoretic pattern of the CNBr-cleaved peptides, (4) the quantitative and qualitative immunological properties, and (5) the catalytic properties. In addition, it was found that the CD profiles in the far ultraviolet wavelength region of the component enzymes were practically identical (data not shown). All these results strongly suggest that the protein structures, both covalent and steric structures, of the component enzymes are identical.

The latter possibility that the microheterogeneity arises from the variation in the carbohydrate moiety of the enzyme is worthy of consideration. The component enzymes were shown to possess a variety of carbohydrate contents (Table I). Especially the amount of the major neutral sugar component, mannose, was found to differ depending on the isoelectric points of the component enzymes; and this difference can be mainly ascribed to a particular peptide obtained by CNBr-cleavage (Figs. 6 and 7). Although it seems hardly conceivable that the isoelectric point of a glycoprotein is determined by the content of a neutral sugar component, there are several examples which imply this possibility [30]. In such cases, differences in the structures of the carbohydrate moieties may affect some dissociable groups of the neighboring amino acid residues to varying degrees, resulting in the observed isoelectric multiplicity.

It should also be taken into consideration that the difference in the isoelectric point might be brought about by the presence and absence of charged groups, such as phosphate, sulfate or amide groups, attached either to the protein moiety or to the carbohydrate moiety of a glycoprotein. In the present case, however, we could detect neither the phosphate or sulfate group in any of the isolated component enzymes, nor find any significant difference in the amide contents. Accordingly, the possibility that the isoelectric multiplicity

comes from these causes is considered very unlikely.

As hitherto described, various methods, as many as possible, have been applied to differentiate the multiple components of glucose oxidase. On the basis of the results, our tentative conclusion is that the microheterogeneity found is most probably due to the difference in the content of the neutral sugar component, mannose, and the structures of the carbohydrate moiety rather than to the difference in the protein moiety.

References

1 Kusai, K. (1960) Ann. Rep. Sic. Works, Fac. Sci. Osaka Univ, 8, 43—74
2 Bodmann, O. and Walter, M. (1965) Biochim. Biophys. Acta 110, 496—506
3 Pazur, J.H. and Kleppe, K. (1965) Biochemistry 3, 578—583
4 Swoboda, B.E.P. and Massey, V. (1965) J. Biol. Chem. 240, 2209—2215
5 Nakamura, S. and Fujiki, S. (1968) J. Biochem. 63, 51—58
6 Abalikhina, T.A., Morozkin, A.D., Bogdanov, V.P. and Kaverzneva, E.D. (1971) Biokhimiya 36, 191—198
7 Nakamura, S., Hayashi, S. and Koga, K. (1976) Biochim. Biophys. Acta 445, 294—308
8 Pazur, J.H., Kleppe, K. and Cepure, A. (1965) Arch. Biochem. Biophys. 111, 351—357
9 Bogdanov, V.P., Abalikhina, T.A., Chukhorva, A.I., Degtyar', R.G. and Kaverzneva, E.D. (1973) Biokhimiya 39, 771—777
10 Hayashi, S. and Nakamura, S. (1976) Biochim. Biophys. Acta 438, 37—48
11 Hayashi, S. and Nakamura, S. (1979) 11th Int. Congr. Biochem. Abstr. 03-5-S18
12 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
13 Doerr, P. and Chrambach, A. (1971) Anal. Biochem. 42, 96—107
14 Vesterberg, O. (1971) Biochim. Biophys. Acta 243, 345—348
15 Zaccharius, R.M., Zell, T.E., Morrison, T.H. and Woodlock, J.J. (1969) Anal. Biochem. 30, 148—152
16 Simpson, R.J., Neuberger, M.R. and Liu, T.-Y. (1976) J. Biol. Chem. 251, 1936—1940
17 Dubois, M., Gilles, K.A., Hamilton, J.K., Reber, P.A. and Smith, F. (1956) Anal. Biochem. 28, 350—356
18 Sweeley, C.C., Bentley, R., Makita, M. and Wells, W.W. (1963) J. Am. Chem. Soc. 85, 2497—2507
19 Aminoff, D. (1961) Biochem. J. 81, 384—392
20 Hartley, B.S. and Massey, V. (1956) Biochim. Biophys. Acta 21, 58—70
21 Spackman, D.H., Smith, E.L. and Brown, D.M. (1955) J. Biol. Chem. 212, 255—269
22 Ambler, R.P. (1967) Methods Enzymol. 11, 436—445
23 Ouchterloney, O. (1958) Prog. Allergy 5, 1—78
24 McDuffie, F.C. and Kabat, E.A. (1953) J. Immunol. 77, 193—197
25 O'Malley, J.J. and Weaver, J.L. (1972) Biochemistry 11, 3527—3532
26 Hayes, M.B. and Wellner, D. (1969) J. Biol. Chem. 244, 6636—6644
27 Plummer, T.H., Jr. and Hirs, C.H.W. (1963) J. Biol. Chem. 238, 1396—1401
28 Plummer, T.H., Jr. and Hirs, C.H.W. (1964) J. Biol. Chem. 239, 2530—2538
29 Reinhold, V.N., Dunne, F.T., Wriston, J.C., Schwarz, M., Sarda, L. and Hirs, C.H.W. (1968) J. Biol. Chem. 248, 6482—6494
30 Montgomery, R. (1972) in Glycoproteins (Gottschalk, A., ed.), Part A, pp. 518—528, Elsevier, Amsterdam