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Simultaneous Immobilization of Glucose Oxidase and Peroxidase to Urea Derivative of Regenerated Acetylcellulose Granules

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ABSTRACT

A method for simultaneous covalent immobilization of glucose oxidase and peroxidase with previously oxidized carbohydrate residues to urea derivative of regenerated acetylcellulose granules is described. The effect of immobilization on the catalytic properties of the separately immobilized enzymes are studied. The immobilized enzymes manifested no change in their pH and temperature optima and slight increase of K_m compared to data for the soluble enzymes. A column packed with simultaneously immobilized enzymes is used for manual glucose determination in blood sera. The results are in high correlation with those obtained by the Beckman Glucose Analyzer method (r = 0.976). The method is economic (the enzyme-carrier conjugate may be used more than 300 times), easy to perform, and less time consuming than the manual methods utilizing soluble enzymes. The established manual method can be proposed for emergency clinical analysis and smaller clinical laboratories.

Index Entries: Simultaneous immobilization; glucose oxidase; peroxidase; determination of glucose; urea derivative of cellulose.

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INTRODUCTION

The immobilization of glucose oxidase and peroxidase to different carriers is of particular interest because of their application for glucose measurements in biological fluids. More successful in aspects of stability and reproducibility is the analysis with covalent enzyme immobilization (*1*). Different granulated carriers and immobilization techniques, by means of chemical modification of amino acid residues (*2,3*) or carbohydrate moiety of these enzymes were used (*4,5*).

A method for covalent binding of enzymes to cellulose carriers was described previously by Krysteva et al. (*6*), which included activation of the cellulose matrix using urea and formaldehyde. The obtained derivative contains active hydroxylmethyl groups, which can bind the amino acid residues of proteins. This method was used successfully for immobilization of some enzymes (*7*) and for preparing multienzyme polysaccharide membranes for biosensors (*8,9*).

For automated analysis of glucose, an enzyme column has been used containing glucose oxidase and peroxidase immobilized simultaneously onto alkylamine glass beads via oxidation of enzyme carbohydrate residues (*10*). This is the only report on simultaneous immobilization of both enzymes on an inorganic carrier. To our knowledge the simultaneous immobilization of glucose oxidase and peroxidase on organic carriers has not been described to date.

The aims of the present work were to immobilize simultaneously glucose oxidase and peroxidase with previously oxidized carbohydrate residues to urea derivative of regenerated acetylcellulose granules, to study the catalytic properties of the biocatalyst produced, and to evaluate the possibility for its use for manual determination of glucose in clinical practice.

MATERIALS AND METHODS

Materials

Glucose oxidase [EC 1.1.3.4] from *Penicillium chrysogenum* was supplied by the Plant of Microbial Preparation in Peshtera (Sofia, Bulgaria). Peroxidase [EC 1.11.1.7] from horseradish was purchased from Reanal (Budapest, Hungary). The commercial preparation of glucose oxidase was further purified by dissolving it in water, followed by dialysis against 50 m*M* phosphate buffer, pH 6.0, to eliminate low-mol-wt substances, and finally by lyophilization. The enzyme peroxidase was used without further purification. Glucose (anhydrous) was supplied by Chemapol (Prague, Czech Republic). 4-Aminophenazone, phenol, and formaldehyde were received from Merck (Darmstadt, Germany). Color reagent (2 m*M* 4-aminophenazone and 14 m*M* phenol in 0.1*M* phosphate buffer, pH 7.0) was pre-

pared following a method described elsewhere (*11*). Control lyophilized sera Precinorm U and Precipath U were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). A transparent triacetylcellulose was used as a matrix for immobilization of the enzymes, produced by treating waste film tapes as described in ref. *12*. All other chemicals used were of reagent grade or better and available commercially.

Methods

Activation of Cellulose Matrix

Granules from triacetylcellulose were obtained according to Chen and Tsao (*13*), as 2 g of transparent were dissolved in solution of dimethylformamide-acetone (ratio 3:2 v/v). The granules with sizes of 0.6–0.8 mm were formed in water bath or in water paraffin (1:1 v/v) bath. The obtained cellulose acetate beads were previously hydrolyzed in 0.1*M* KOH for 24 h in order to de-esterify the acetyl groups. The urea derivative was then prepared as follows: 2 g of hydrolyzed cellulose granules were treated with sodium periodate (0.25 m*M* at a temperature of 20°C and pH 5.0 for 2 h in darkness). Upon completion of the process, the beads were washed with distilled water in a Buchner funnel until there was no periodate left. The oxidized cellulose granules were then immediately treated with 15% (w/w) urea for 16 h in the presence of 0.9% (v/v) sulfuric acid at 60°C. The granules were then washed with distilled water in a Buchner funnel until the rinsings showed a neutral reaction. The resulting urea derivative of the cellulose contained 5.27% nitrogen.

Simultaneous Immobilization of Glucose Oxidase and Peroxidase with Oxidized Carbohydrate Residues

The oxidation of carbohydrate residues of glucose oxidase and peroxidase with periodic acid (0.04 m*M* in 0.05 m*M* acetate buffer, pH 5.0) was performed according to the method of Zaborsky and Ogletree (*5*). The unreacted periodic acid was removed with 0.025 mL ethylene glycol. The oxidized enzymes were dialyzed against 50 m*M* phosphate buffer with pH 6.0 for 18 h. The binding of both oxidized glycoenzymes to urea derivative of cellulose beads was performed in 0.1 m*M* acetate buffer, pH 3.8, for 18 h at 4°C, with careful magnetic stirring. The activated granules for the individual binding of the enzymes were separately treated with 90 mL enzyme solution containing 9 mg/mL glucose oxidase or peroxidase. The enzyme solution for the simultaneous binding of the enzymes to the activated cellulose matrix consisted of 9 mg/mL and 3 mg/mL glucose oxidase and peroxidase, respectively. The obtained carrier-enzyme conjugate was carefully washed with distilled water until no absorbance was observed at 280 nm in the rinsing water. The immobilized enzymes were stored in a moist state at 4°C.

Enzyme Activity Assay

The activities of glucose oxidase and peroxidase were determined using Trinder-reagent (*14*). The increase in absorbance at 500 nm was measured using a Spekol 11 spectrophotometer (Zeiss, Jena, Germany).

One unit of glucose oxidase activity was taken to be that amount of enzyme which caused the release of 1 μmol of hydrogen peroxide per 1 min at a temperature of 25°C and at pH 6.0. One unit of peroxidase activity was defined as that amount of enzyme which caused the decomposition of 1 μmol of hydrogen peroxide per 1 min at 25°C and at pH 7.0.

The activities of glucose oxidase and peroxidase used to perform the binding were 52 U/mg and 245 U/mg, respectively.

The amount of bound protein was determined using a modified Lowry's method (*15*). Bovine serum albumin was used as a standard.

Determination of the pH-Optima and Temperature Optima

The residual activities of soluble and immobilized enzymes were determined in 0.1 m*M* phosphate buffer (for the range of pH 5.0–8.0) at 25°C and for the temperature range of 20–40°C at pH 6.0 (for glucose oxidase) and pH 7.0 (for peroxidase).

Determination of Michaelis' Constants

The Michaelis' constants, K_m, of native and immobilized enzymes were determined at increasing substrate concentrations in 0.1 m*M* phosphate buffer at the optimal pH (0.1–10 m*M* glucose for glucose oxidase and 5–20 m*M* hydrogen peroxide for peroxidase). The K_m values were calculated using a computer program.

Determination of Glucose

Using An Immobilized Multienzyme Column

The immobilized multienzyme complex (about 0.25 g dry wt) was packed in a little glass column thermostated at 37°C (25 mm in length and 10 mm in inner diameter), as previously described (*16*). An assay solution containing 0.2 mL sample (control or blood serum or glucose solution in 0.1 m*M* phosphate buffer with pH 6.0) and 0.2 mL color reagent solution were introduced together into the column. After a 5-min incubation at 37°C, 4 mL distilled water was added and the liquid was eluted with a vacuum pump. The resulting absorbance of the dyed product was measured through a glass cuvet with a 1 cm light path at 500 nm. After washing with 5 mL 0.1 m*M* phosphate buffer at pH 6.0, the column was ready for storage or further assays. The glucose content (in m*M*) was calculated using a calibration curve.

Statistical Methods

The results were evaluated by the methods of descriptive statistics and linear regression analysis (*17*). Simple regression analysis of measured glucose concentrations was accomplished utilizing the computer program StatView 512+,™ from Brain Power, Calabasas, CA.

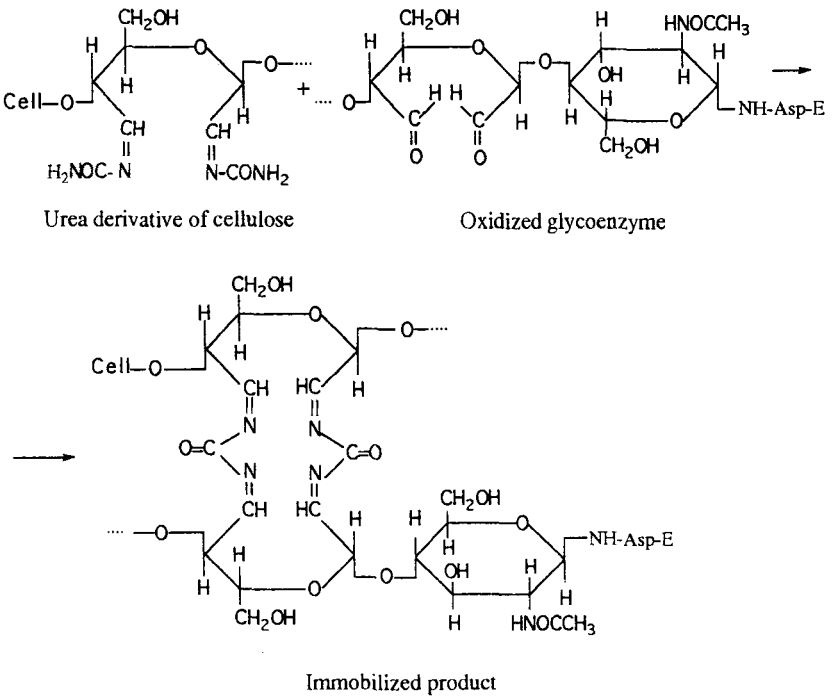

Fig. 1. Scheme of covalent binding of the oxidized glucose oxidase and peroxidase to urea derivative of regenerated acetylcellulose granules.

RESULTS AND DISCUSSION

An artificial multienzyme complex was prepared in which glucose oxidase and peroxidase were covalently bound to activated cellulose granules. The simultaneous immobilization of glucose oxidase and peroxidase on the cellulose carrier with amide groups was performed after mild periodate oxidation of vicinal hydroxyl groups of their carbohydrate parts able to make active aldehyde groups. The proposed method of enzyme immobilization with oxidized carbohydrate residues to urea derivative of cellulose probably involved the reaction in Fig. 1.

The study of coupling reaction showed that maximum immobilization of enzyme activity was achieved with lower pH solution. This might be because the acidic pH favored the reaction between amide groups of carrier and aldehyde groups from oxidized carbohydrate moieties of the enzymes. Coupling of oxidized glycoenzyme with urea derivative of cellulose resulted in an active enzyme conjugate, presumably through the imine linkage. The protein loading with the carrier was about 28–34 mg enzyme per g of enzyme conjugate (Table 1). A higher degree of condensation was achieved at pH 3.8–4.0. This correlated to higher activity, obtained for bound enzymes. The accomplishment of the reaction in the acidic pH region represented an advantage of this method, since glucose

Table 1
Catalytic Properties of the Immobilized Enzymes

Enzyme	Amount of bound protein, mg/g dry carrier	pH optimum	Temp. optimum, °C	Specific activity, U/mg	Relative activity, %	Activity, U/g dry carrier	K_m m*M*
Glucose oxidase	34	5.8(5.8)[a]	30(30)	23(52)	45	782	1.8(1.5)
Peroxidase	32	7.0(7.0)	35(35)	146(245)	60	4672	12.3(11.9)
Simultaneously immobilized glucose oxidase and peroxidase	28	–	–	–	–	610 2200	–

[a] The figures in parentheses refer to the free enzymes.

oxidase, isolated from *Penicillium chrysogenum* (*18*), was not stable and its activity decreased rapidly at the alkaline pH values.

The advantage of glycoenzyme immobilization via their carbohydrate moieties resulted because the covalent bonds always occurred off the active site of the enzyme, thus enabling the enzyme to sustain a high degree of its original activity (Table 1). The obtained conjugates showed a good stability. After two months storage at 4°C, the activity of immobilized enzymes was not practically decreased.

It has been established (*10*) that the highest sensitivity of glucose determination can be received at an activity ratio of 3:1 between glucose oxidase and peroxidase. Therefore, both enzymes were mixed at the same ratio of activities (3:1) prior to their immobilization.

The immobilized enzymes were characterized regarding their pH and temperature maxima, K_m, and then were compared with those of the free enzymes (Table 1).

As shown on Table 1, the enzymes manifested no change in their pH and temperature optima and showed a slight increase of their Michaelis' constants. The immobilized glucose oxidase and peroxidase showed the highest activity at pH 5.8 and 7.0, respectively. The preservation of the pH optima of the immobilized enzymes showed, on one hand, that the uncharged cellulose matrix did not influence the enzymes molecules and, on the other hand, that there were not diffusion limits to the reaction. The temperature optima for both immobilized enzymes, glucose oxidase and peroxidase, were at 30°C and 35°C, respectively. The close K_m values of both soluble and immobilized enzymes showed that immobilization had an insignificant effect on the enzyme-substrate interactions.

The results can be interpreted based on the influence of the immobilization procedure on the bound enzyme. Glucose oxidase and peroxidase are glycoproteins, which are composed of 16% (Mr 150,000) (*19*) and 18% (Mr 40,000) (*20*) carbohydrate residues, respectively. Some previous investigations showed that these enzymes have been bound successfully to granulated synthetic carriers (*5*), Sepharose, or alkylamine glass beads (*11,21*) by means of their oxidized carbohydrate residues. The present novel approach for covalent binding of glucose oxidase and peroxidase to urea derivatives of cellulose granules tends to show higher values of bound protein when compared to methods utilizing other carriers (*5,11*) (28–34 mg/g vs 6–19 mg/g protein) and also showed a high degree of retained activity (Table 1).

Although most estimations of blood glucose are now performed by automated methods, a need remains for occasional manual analysis of blood sera, particularly in emergency clinical practice and for work in small clinical laboratories. The column packed with simultaneously immobilized glucose oxidase and peroxidase to the urea derivative of cellulose was successfully used for manual analysis of glucose in sera. The use of immobilized enzymes in this form is attractive mainly because of the stability of the product, which makes it possible to utilize the same conjugate

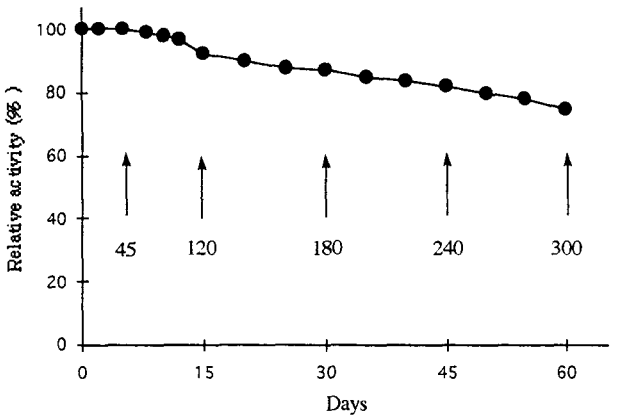

Fig. 2. Operational stability of simultaneously immobilized glucose oxidase and peroxidase in multienzyme column. The numerals with an arrow indicate cumulative number of serum samples assayed through the same column.

more than 300 times and was therefore more economical. The multienzyme column reactor showed a good operational stability within two months of use (Fig. 2). As seen on Fig. 2, the initial activity of the simultaneously immobilized enzymes decreased by 22% after being reused for 60 d, which allowed a good reproducibility of the measurements. Linearity was excellent, not only with aqueous glucose solution, but also with reference serum samples in the glucose concentration range of 1–15 m*M*. A linear response was obtained even when the activity of the multienzyme column decreased to about 20% of the initial values. An additional advantage of the manual method described here is that the enzymic determination of glucose can be performed in only 5 min, which is several times shorter than the other routine manual methods utilizing soluble enzymes, which required more than 20 min for the complete performance (*22*).

The multienzyme column allows an accurate estimation of gluose in blood sera. The within-run precision (*17*) of the analysis of glucose was evaluated by calculation of the coefficient of variation (CV = 3.71%) after measuring 20 consecutive samples of lyophilized control serum (mean value of 7.0 m*M* glucose). The day-to-day precision was estimated by calculation of the coefficient of variation (CV = 4.17%) after analyzing samples of control serum (mean value of 7.20 m*M* glucose) during 20 d, in single determination each day (Table 2). A CV below 4.3% has been accepted as a requirement to glucose determination in reference laboratories (*23*). Since the present method resulted in an intra-assay CV of 3.71% and an interassay CV of 4.17%, we can conclude that it offers good precision and reproducibility of the measurements and can be further used in laboratory practice.

To estimate the precision of the proposed manual method, we performed simultaneous measurements of Beckman Glucose Analyzer 2 that utilized soluble glucose oxidase. The Beckman method is the most widely applied method for the analysis of glucose in blood sera. Blood specimens

Table 2
Precision of Glucose Measurements (n = 20) in Control Serum Using Multienzyme Column

Serum samples	Within-run (intra-assay) precision	Day-to-day (inter-assay) precision
X[a], [m*M*]	7.00	7.20
S[b], [m*M*]	0.26	0.30
CV, %	3.71	4.17

[a]Mean values.
[b]Standard deviation.

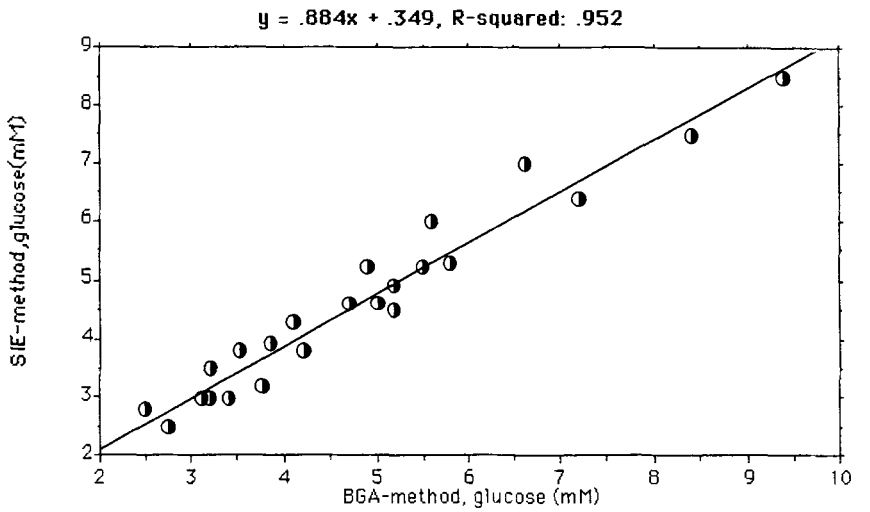

Fig. 3. Correlation between glucose concentrations in blood serum as measured by the simultaneously immobilized glucose oxidase and peroxidase method (SIE-method) and by the soluble glucose oxidase method using Beckman glucose analyzer (BGA-method). Concentration of glucose from the BGA-method is assigned to the x-axis (as independent variable) and the y-axis represents the concentration of glucose from the SIE-method (as dependent variable).

were taken from 24 patients with different blood glucose levels ranging from 2.5–9.5 m*M*. Data obtained by both methods were compared using simple regression analysis. The results were plotted on a regression line (Fig. 3), with the glucose concentrations from the present method assigned to the y-axis (SIE-method), and the glucose concentrations from the Beckman method to the x-axis (BGA-method). The obtained regression equation is:

$$y = 0.884\,(\pm 0.042)\,x + 0.349$$
$$n = 24\, r = 0.976\, F_{1,22} = 434.4\, (F_{1,22;\,p\,=\,0.05} = 4.30)$$

where r is the correlation coefficient. The employed significance test is F-test at confidence level $p = 0.05$. A good correlation existed between the results of our method and those from the referent Beckman method. Data from all statistical calculations confirmed the precision of the proposed method.

Stability of the investigated column was very high, comparable with that of simultaneously immobilized enzymes onto alkylamine glass beads (*10*). The attachment of both glucose oxidase and peroxidase to the carrier was achieved by means of oxidation of carbohydrate residues outside their active site. Thus, the immobilization did not lead to conformational changes in the enzyme active site and resulted in preservation of a high enzymatic activity, insensitivity towards enzymatic deactivation and guaranteed kinetic control of the velocity of the enzyme reaction in the column.

CONCLUSION

This study shows that glucose oxidase and peroxidase with preliminarily oxidized carbohydrate residues can be successfully immobilized simultaneously on urea derivative of regenerated acetylcellulose. The novel multienzyme granulated system offers several advantages: The obtained cellulose carrier is cheap and easy to cast in granulated form; binding of enzymes is simple to carry out and the obtained multienzyme system shows high stability and activity; and the immobilized enzymes demonstrated excellent capability for practical application and could be used to measure glucose in sera or in fermentation broths and foods. The proposed manual multienzyme column method can be recommended for emergency clinical analysis and smaller clinical laboratories.

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REFERENCES

1. Scouten, W. H. (1987), in *Methods in Enzymology*, vol. 135, part B, Mosbach, K., ed., Academic, New York, pp. 30–64.
2. Foulds, N. C. and Lowe, C. R. (1988), *Anal. Chem.* **60,** 2473–2478.
3. Hossain, Md. M. and Do, D. D. (1985), *Biotechnol. Bioeng.* **27,** 842–851.
4. Royer, G. P. (1987), in *Methods in Enzymology*, vol. 135, part B, Mosbach, K., ed., Academic, New York, pp. 141–146.
5. Zaborsky, O. R. and Ogletree, J. (1974), *Biochem. Biophys. Res. Commun.* **61,** 210–216.

6. Krysteva, M. A., Blagov, S. R., and Sokolov, T. T. (1984), *J. Appl. Biochem.* **6,** 367–373.
7. Krysteva, M. A. and Zlateva, T. P. (1988), *Biotechnol. Appl. Biochem.* **10,** 124–130.
8. Entcheva, E.G. and Yotova, L. K. (1994), *Anal. Chim. Acta* **299,** 171–177.
9. Krysteva, M. A. and Yotova, L. K. (1992), *J. Chem. Tech. Biotechnol.* **54,** 13–18.
10. Murachi, T., Sakaguchi, T., Tabata, M., Sugahara, M., and Endo, J. (1980), *Biochimie* **62,** 581–585.
11. Endo, J., Tabata, M., Okada, S., and Murachi, T. (1979), *Clin. Chim. Acta* **95,** 411–417.
12. Krysteva, M. A., Peev, G. A., and Yotova, L. K. (1987), *Acta Biotechnol.* **7,** 93–96.
13. Chen, L. F. and Tsao, G. T. (1976), *Biotechnol. Bioeng.* **18,** 1507–1516.
14. Trinder, P. (1969), *J. Clin. Path.* **22,** 246.
15. Schacterlle, G. R. and Pollack, R. L. (1973), *Anal. Biochem.* **51,** 654–655.
16. Ivanov, I. P., Danev, S. I., Palashev, Ch. D., and Dimitrov, D. G. (1990), *Lab. Delo.* (Russ) **1,** 29–31.
17. Miller, J. C. and Miller, J. N. (1994), *Statistics for Analytical Chemistry.* 3rd ed., Ellis Horwood and Prentice Hall, New York.
18. Kojuharova, A., Popova, J., and Kirova, N. (1988), *J. Chem. Technol. Biotechnol.* **42,** 95–104.
19. Pazur, J. H., Kleppe, K., and Cepure, A. (1965), *Arch. Biochem. Biophys.* **11,** 351–357.
20. Striclanes, E. A., Hardine, E. K., Kay, F. E., Shanom, L. M., and Horwitz, J. (1968), *J. Biol. Chem.* **243,** 3560–3564.
21. Hsiao, T. and Rower, H. (1979), *Arch. Biochem. Biophys.* **198,** 379–382.
22. Bauminger, B. B. (1974), *J. Clin. Path.* **27,** 1015–1017.
23. Stamn, D. (1986), *Z. Lebenm. Forsch.* **182,** 372–384.