0273-2289/93/4303—0177$03.40

Protease Determination Using an Optimized Alcohol Enzyme Electrode

G. BARDELETTI* AND C. CARILLON

Laboratoire de Biochimie Biotechnologie, EP19 CNRS, Bat 303, Université Claude Bernard-Lyon1, 43 boulevard du 11 Novembre 1918, 69622, Villeurbanne Cedex, France

Received February 2, 1993; Accepted May 11, 1993

ABSTRACT

A new method for the determination of protease activities is described. In this large family, trypsin is used as a protease model that cleaves the ethyl or methyl ester of artificial substrates producing ethanol or methanol. Alcohol is detected using an alcohol oxidase enzyme electrode. The H_2O_2 production that occurs is measured amperometrically. At 30°C, in a 0.1*M* phosphate buffer, pH 7.5, the enzyme electrode response for ethanol was calibrated at 3.10^{-6}–$3.10^{-3}M$ and for methanol from 3.10^{-7} to $4.10^{-4}M$ in the cell measurement. Trypsin levels as determined by the proposed method and by a conventional spectrophotometric method are in good agreement when using the same measurement conditions. A detection limit of 10 $U \cdot L^{-1}$ and a linear calibration curve of 10–100,000 $U \cdot L^{-1}$ in the sample were obtained. Measuring time for the required trypsin solution concentration was from 4 min (for the most dilute samples) to 1 min (for the most concentrate samples). In a typical experiment, protease measurements did not inactivate the alcohol oxidase on the probe, nor did a more classical use for alcohol detection. The procedure developed could permit any protease estimation on the condition that they hydrolyze ester bonds from synthetic substrate.

Index Entries: Amperometric enzyme electrode; ethanol; methanol; protease activity; trypsin.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Enzymes have played an important role as analytical reagents in the development of biosensors in the areas of clinical and biotechnological analysis. The use of an enzyme in association with an electrode was the first type of biosensor proposed. This technology combines the specificity of an enzymatic reaction with the analytical power of an electrochemical detection applied for the first time to glucose determination (*1*). Since then, a strong interest in such analytical devices arose, and numerous papers, reviews, and books have appeared in the literature describing enzyme electrodes and uses (*2–9*). However, considerable effort is still under way to develop reliable, accurate, convenient, low-cost, and low-maintenance analyzers (*10*).

Significant improvements have recently been proposed by utilizing new types of enzyme-sensor associations, novel supports or immobilization procedures, more appropriate electrochemical detection techniques, or by using novel enzymes (*11*). However, several drawbacks must still be overcome for the wider utilization of such devices, and despite extensive efforts by many researchers, applications were very slow.

Several enzyme electrodes are reported for alcohol detection principally for ethanol and methanol. The most common transducers used in the design of these electrodes are based on amperometric measurement of H_2O_2 production (*5,12–14*) or O_2 depletion (*15–18*) involved in the reaction using oxidase enzymes or microorganisms. Other alcohol determination methods involve chemically modified electrodes (*19–21*), or thermistor probes in a flow system (*22*) or an all solid-state sensor using alcohol oxidase and β-NAD^+ (*23*). An application of these alcohol sensors for the determination of an artificial sweetener (aspartame) was recently reported (*24*).

Routinely, enzyme activities in biological media are determined by spectrophotometric methods where expensive reagents, such as coenzymes (NAD(P)H), are necessary. Usually, these methods require sample pretreatments, and the procedure is time-consuming. Only recently, a few reports have dealt with the use of biosensors for the measurement of enzyme activities that have the advantage of being convenient and inexpensive (*25–27*).

This article describes the use of a nonspecific biosensor to measure protease activities of a large enzyme family using trypsin as a working model. The activity of this enzyme family has never been determined using biosensors, even though proteases are one of the most important industrial enzymes (59%) as biological detergents, tanning agents, and in the food industry: backing, brewing, cheese manufacturing, and meat tenderizing (*28*). The detection of protease is also of some importance in the cell culture media to keep track of enzyme production or contamination, and could be of some interest in the control of effluent in the waste-water treatment.

MATERIALS AND METHODS

Chemical Products and Instrumentation

Alcohol oxidase (AO, EC 1.1.3.13) from *Pichia pastoris* (10–40 U/mg protein), trypsin from bovine pancreas (10,000–13,000 BAEE U/mg protein), *N*-α-Benzoyl-L-Arginine Ethyl Ester (BAEE), *N*-α-Tosyl-L-Arginine Methyl Ester (TAME), and Flavine Adenin Dinucleotide (FAD) disodium salt were from Sigma Chemical Co. AO from *Candida boidinii* (5 U/mg protein) was from Boehringer Mannheim, France. Methanol, ethanol, 1-propanol, 2-propanol, and butanol were purchased from Merck. All other reagents of the highest available grade were supplied by Prolabo, France. Biodyne immunoaffinity membranes (120 μm thick, 1.2 μm rating) in a preactivated form from Pall Industrie S.A, France, were used for enzyme immobilization. The following electrochemical devices from Solea-Tacussel, France were used: a self-contained enzyme electrode type "Gluc1," here equipped with an AO membrane tightly pressed against the platinum tip (3 mm) with a screw cap and plugged to a polarograph-type PRGE.

Enzyme Immobilization

The immobilization procedure used here was reported previously (*29*). Coupling was achieved at 4°C by simple immersion of 8-mm diameter disks cut from the preactivated membrane in a stirred AO solution of the required concentration in a 0.1*M* phosphate buffer, pH 7.5. After 1 h, the disks were washed twice for 15 min in 1*M* KCl at room temperature and stored at −20°C in a 0.1*M* phosphate buffer, pH 7.5, 0.1*M* KCl with 10% (v/v) glycerol or 30% (w/v) sucrose.

Procedure

The potential of the platinum anode of the probe was fixed at +650 mV vs an Ag/AgCl reference electrode for H_2O_2 detection. The anodic current was output to a Sefram-Servotrace recorder. The enzymic electrode was immersed in a thermostated vessel (30°C) containing 10–20 mL of 0.1*M* phosphate buffer, pH 7.5, 0.1*M* KCl into which alcohol sample, protease substrate, or protease solution were added. In every case, the current output was recorded, and the sensitivity calculated in $mA \cdot M^{-1}$.

For determination of protease, the trypsin solution was added into the measurement cell containing the substrate (TAME or BAEE). The current increased with time owing to the alcohol produced, which is proportional to the enzyme concentration added.

A conventional method, using TAME or BAEE, with the hydrolysis of the ester followed spectrophotometrically at 247 or 255 nm, respectively,

was used as a comparison method for trypsin (*30*). Extinctions were recorded at 25 or 30°C using an Uvikon recorder 21 directly connected to the Uvikon 810 spectrophotometer (Kontron Instruments).

RESULTS AND DISCUSSION

Alcohol Oxidase Coupling Conditions

Enzyme stability is an intrinsic property determined by the structure of the enzyme protein. External environmental factors, including divalent cations, substrates, coenzymes, and some other additives as polyols or proteins, often increase enzyme stability. Many enzymes are more stable in the presence of their substrates or some additives (*31*). The procedures that have been successful in enzyme stabilization are those that prevent unfolding of the protein secondary and tertiary structure, thus maintaining the enzyme in its fully active conformation.

The enzyme of *Candida boidinii* is a flavoprotein containing FAD as a prosthetic group bound noncovalently to the enzyme protein (*32*). Alcohol oxidase from other microorganisms also contains FAD as a prosthetic group (*33*).

In a typical experiment, sensor sensitivity was tested when using alcohol oxidase from *Pichia pastoris* or from *Candida boidinii* (both at 40 $U \cdot mL^{-1}$ in the coupling solution) over definite times for alternate periods of storage (−20°C) and operation (30°C).

Alcohol oxidase seems to denature very quickly, even when immobilized (Fig. 1). So, for a practical and extensive use of an alcohol sensor, several additives and different storage conditions were tested with a view toward improved stabilization.

The influence of variable concentration of the prosthetic group (FAD 0.4–50 μM), alcohol oxidase substrates (methanol or ethanol 1–100 mM), or short polyalcohol (glycerol 0.5–10% [v/v]) in the 0.1M phosphate buffer was tested in the immobilization procedure.

There was no change in the initial sensitivity of the enzyme electrode, but for longer use, the stability of the probe was improved when alcohol oxidase was grafted with some additives in the buffer, and the best result thus obtained is shown in Fig. 1. Optimized conditions for enzyme immobilization, alcohol measurement, and enzymic membrane storage are reported in Table 1.

Selectivity of the Alcohol Biosensor

The substrate selectivity of the alcohol sensor was tested, and the results are shown in Fig. 2. Measurements were carried out for each alcohol at $10^{-4}M$ concentration. The sensor had a wide specificity. Methanol was the primary substrate of the enzyme both from *Candida boidinii* and *Pichia*

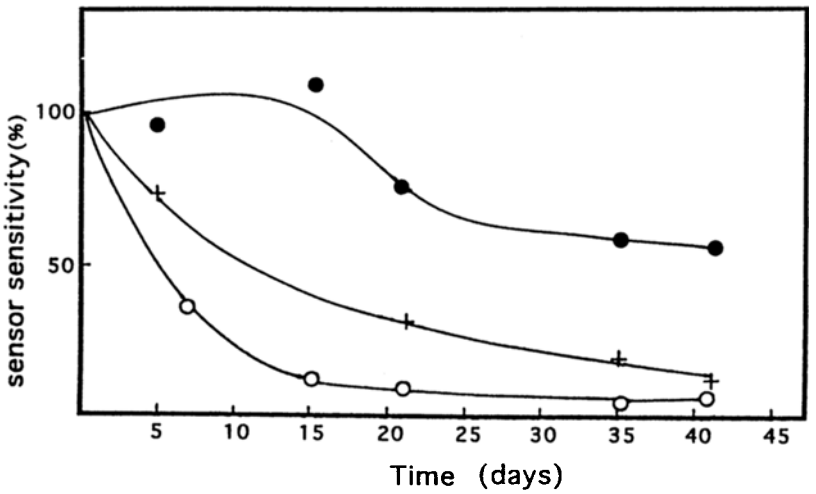

Fig. 1. Evolution of the sensitivity of alcohol enzyme electrode with alcohol oxidase origin and coupling conditions. (+) Enzyme from *Candida boidinii* and immobilization performed at 4°C in 100 m*M* phosphate buffer, pH 7.5, KCl 100 m*M*. (○) Enzyme from *Pichia pastoris* and immobilization performed at 4°C in 100 m*M* phosphate buffer, pH 7.5, KCl 100 m*M*. (●) Enzyme from *Pichia pastoris* and immobilization performed at 4°C in 100 m*M* phosphate buffer, pH 7.5, KCl 100 m*M* with additives: 10 m*M* $MgCl_2$, 5.10^{-3} m*M* FAD, 50 m*M* ethanol. The sensor responses were tested over alternate periods of storage (−20°C) and operation (20 alcohol determinations).

Table 1
Optimal Conditions for Alcohol Oxidase Biosensor

Parameter	A.O. immobilization *Pichia pastoris*	A.O. membrane conservation	Alcohol measurement
Buffer	100 m*M* phosphate pH 7.5	100 m*M* phosphate pH 7.5	100 m*M* phosphate pH 7.5
KCl	100 m*M*	100 m*M*	100 m*M*
$MgCl_2$	10 m*M*		
FAD	5.10^{-3} m*M*		
Ethanol	50 m*M*		
Glycerol		10%	
Temperature	4°C	−20°C	30°C

pastoris starting from the same concentration (40 $U \cdot mL^{-1}$) in the coupling buffer. Responses obtained using alcohol oxidase from *Picha pastoris* were seven- to eightfold higher than those obtained using *Candida boidinii* enzyme (for methanol, initial sensitivities were between 0.7–0.8 and about 0.1 $mA \cdot M^{-1}$, respectively). This higher specific activity, using alcohol oxidase from *Pichia pastoris*, has also been reported by Danielson (*34*) with an enzyme thermistor device.

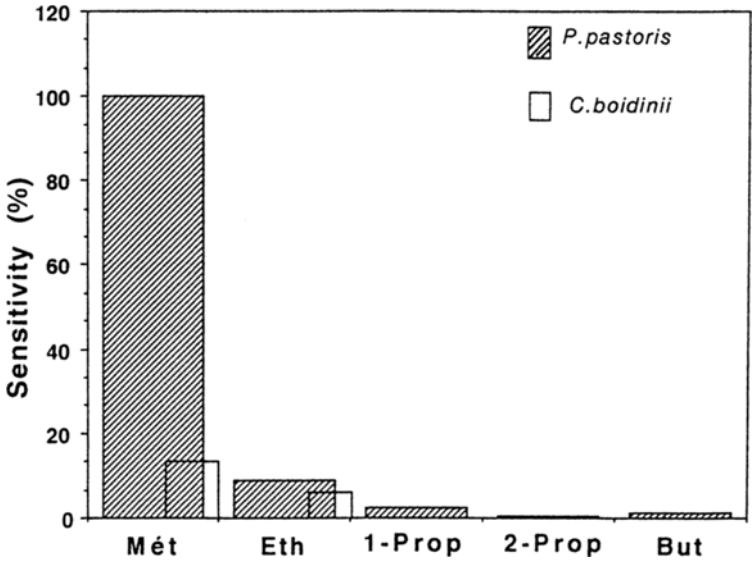

Fig. 2. Comparison of the enzyme electrode response to alcohols. Each alcohol at $10^{-4}M$ concentration, measurements performed at 30°C in 100 m*M* phosphate buffer, pH 7.5, KCl 100 m*M*.

Effect of pH

The effect of pH on the enzyme electrode using A.O from *Pichia pastoris* was investigated at 30°C over the pH range 6.5–8 at $10^{-4}M$ ethanol and $10^{-5}M$ methanol. The results are displayed in Fig. 3. The maximum sensitivity (0.65 mA · M^{-1} for methanol and 0.08 mA · M^{-1} for ethanol) is observed at pH 7.5. Below pH 7, sensitivity decreases rapidly. This result is in good agreement with those obtained by Nanjo and Guilbault (*12*) using an alcohol oxidase from the *mycelium* of a *Basidiomycete* and by Belgith et al. (*13*) using the enzyme from *Hansenula polymorpha*.

Temperature Dependence

In order to assess the temperature dependence of the sensor, methanol and ethanol determinations were done at several temperatures between 21 and 35°C for 1.10^{-5} and $1.10^{-4}M$ concentrations, respectively. As shown in Fig. 4, sensitivity varied strongly with temperature, the greatest values being obtained at 30°C (0.74 mA · M^{-1} for methanol and 0.065 mA · M^{-1} for ethanol). Using higher temperatures, response leveled off quickly.

Calibration Plot and Threshold

To determine the linear range, methanol and ethanol solutions were tested. The calibration graph on a double logarithmic scale is shown in

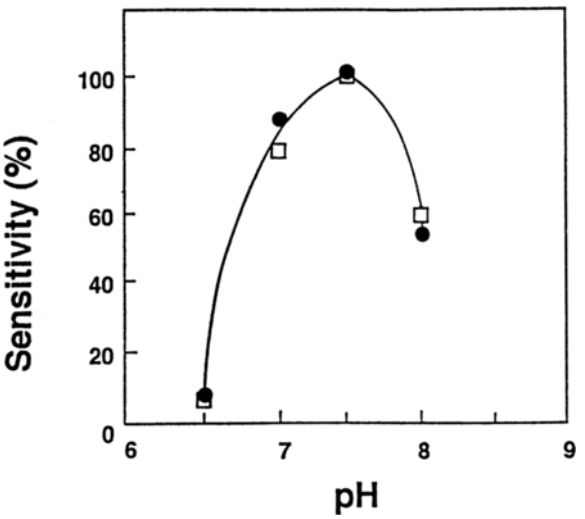

Fig. 3. Effect of pH on the alcohol enzyme electrode sensitivity to (□) $10^{-5}M$ methanol and (●) $10^{-4}M$ ethanol. Measurements performed at 30°C in 100 m*M* phosphate buffer, KCl 100 m*M*; A.O from *Pichia pastoris.*

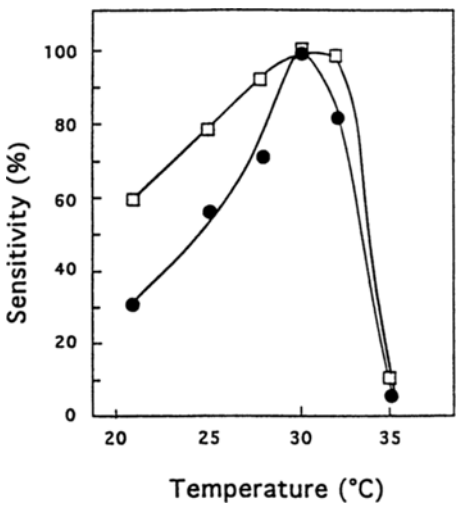

Fig. 4. Effect of temperature on alcohol enzyme electrode sensitivity to (□) $10^{-5}M$ methanol and (●) $10^{-4}M$ ethanol. Measurements performed at 30°C in 100 m*M* phosphate buffer, pH 7.5, KCl 100 m*M*; A.O from *Pichia pastoris.*

Fig. 5. Responses to methanol concentration of 0–0.1 μM were not significantly above the noise level (usually lower than 10 nA) and were not detectable. The threshold was $2.10^{-7}M$, and a linear calibration range from 3.10^{-7} to $4.10^{-4}M$ was routinely obtained. Responses to ethanol were less sensitive. The threshold was $1.10^{-6}M$, with an accurate measurement and calibration linearity obtained between 3.10^{-6} and $3.10^{-3}M$. In both cases,

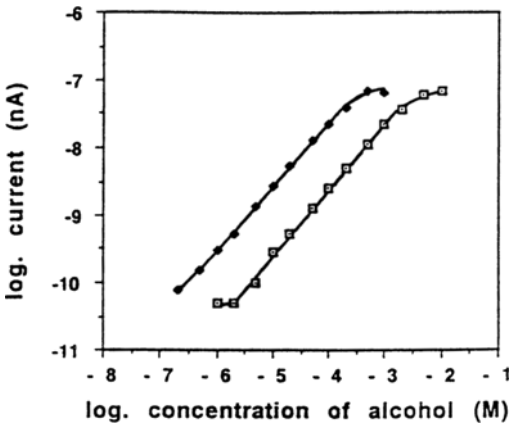

Fig. 5. Calibration curves of the alcohol enzyme electrode to ethanol (□) and methanol (◆). Measurements performed at 30°C in 100 m*M* phosphate buffer, pH 7.5, KCl 100 m*M*; A.O from *Pichia pastoris*.

at higher concentration, the response leveled off. The relative standard deviations for 10 replicate assays of $1.10^{-5}M$ methanol or $1.10^{-4}M$ ethanol were 1.5 and 2%, respectively.

Protease Determination

Trypsin hydrolyzes bonds in proteins and peptides involving the carboxyl groups of lysine or arginine. Peptide links are not necessary for hydrolysis. Amides are split more rapidly than peptide, and esters are split even more rapidly. The rate of hydrolysis of the ester is not affected by the nature of the alcohol group (*35*), so, to determine trypsin activity, the amides or esters of arginine (BAEE or TAME) or lysine were largely used (*36*). The hydrolysis of these substrates can be followed spectrophotometrically. Measurement using *N*-α-*p*-Toluenesulfonyl-L-Arginine methyl ester as substrate was achieved at 247 nm in an 8.1 optimum pH buffer. Determination of activity using *N*-α-Benzoyl-L-Arginine ethyl ester was achieved at 255 nm using the same buffer; in both cases, the temperature is 25°C. Results can be expressed by the activity unit based on the volume ($U \cdot L^{-1}$) or on the weight ($U \cdot mg^{-1}$). One unit is the enzyme activity that transforms 1 μmol of substrate in 1 min under optimal conditions. In sample, the volume activity ($U \cdot L^{-1}$) was calculated according to Bergmeyer et al. (*37*).

Protease was determined with the enzyme electrode using alcohol oxidase from *Pichia pastoris* immobilized on a preactivated permeable membrane using the measurement buffer containing BAEE or TAME. Standard ethanol or methanol solution was added to calibrate the sensor, and then trypsin solution was added. The current increasing with time is proportional to the concentration of alcohol generated by the protease added.

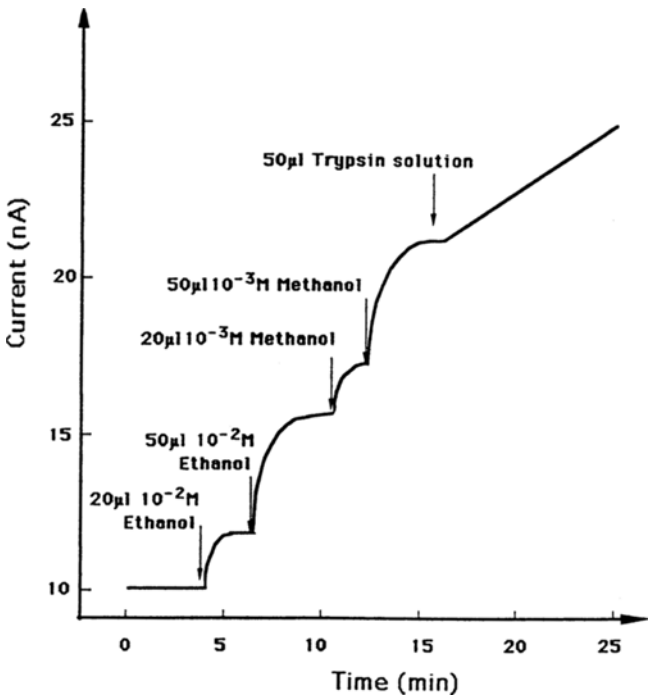

Fig. 6. Typical response of amperometric assay of the protease activity using an alcohol enzyme electrode. Current vs time response curve including calibration by product (ethanol or methanol). Measurements performed at 30°C in 100 m*M* phosphate buffer, pH 7.5, KCl 100 m*M*, BAEE or TAME 1 m*M*; A.O from *Pichia pastoris*. A 50-µL aliquot of trypsin solution is added to 10 mL of the suitable buffer. A 10-nA initial noise level current was routinely obtained using phosphate buffer.

The time-course of a typical process is shown in Fig. 6. Using this procedure, trypsin activity was measured within 4 min. The detection limit for trypsin was 10 U · L^{-1} in the sample. The concentration of a trypsin solution was determined both by the amperometric procedure described here and by a conventional spectrophotometric method. Results are shown in Table 2. Results vary with the substrates and the procedures used (Tris buffer pH 8.1 with $CaCl_2$ at 25°C in the conventional spectrophotometric method and phosphate buffer pH 7.5 without Ca^{2+} at 30°C in the optimized amperometric alcohol measurement). Owing to increased stability of trypsin solutions in the presence of Ca^{2+}, a higher activity of the enzyme is found in most assay systems when Ca^{2+} is added (*30*). Enzyme activities were strongly dependent on the procedure, so we tried to standardize the two procedures. Tris buffer giving a high noise level current was not very useful in amperometric measurements, so we have compared the two procedures using phosphate buffer, pH 7.5, with $CaCl_2$ at 30°C. A good

Table 2
Comparison of Protease Activity as Measured
by the Alcohol Electrode and Spectrophotometric Method

	Alcohol electrode	Spectrophotometric method		
Measurement conditions	0.1*M* Phosphate buffer pH 7.5, 0.1*M* KCl 30°C	0.1*M* Phosphate buffer pH 7.5, 0.1*M* KCl, 15 m*M* $CaCl_2$ 30°C		50 m*M* Tris buffer pH 8.1, 15 m*M* $CaCl_2$ 25°C
Substrate	1 m*M* BAEE or 1 m*M* TAME			
Protease	Trypsin solution			
Results ($U \cdot L^{-1}$)				
BAEE substrate	410	550	580	690
TAME substrate	230	290	310	370

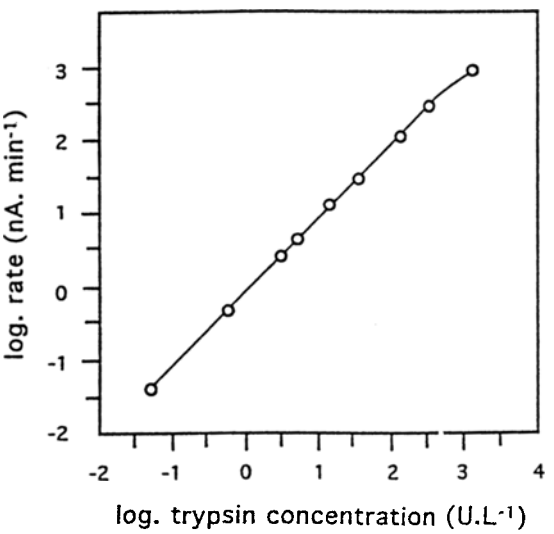

Fig. 7. Calibration curve for trypsin solutions. Measurements performed at 30°C in 10 mL of the optimized conditions of the total reaction system: 100 m*M* phosphate buffer, pH 7.5, KCl 100 m*M*, $CaCl_2$ 15 m*M*, and TAME 1 m*M* (for the last two determinations, 2 m*M*). The indicated trypsin concentrations (0.05–1350 $U \cdot L^{-1}$) were those in the measuring cell, using 20 or 50 μL of 10–500,000 $U \cdot L^{-1}$ trypsin solution samples.

correlation is found between the results from the enzyme electrode and those from a classical procedure (Table 2). It was noticeable that in both procedures the results depended on the substrate choice. Trypsin used in our study hydrolyzed the ester bond from ethanol more quickly than those from methanol. Using the optimized conditions of the total reaction system, including trypsin hydrolysis and alcohol oxidation, detection limit is not improved. The calibration curve performed by using a standard trypsin solution is shown in Fig. 7. A linear range of 10–100,000 $U \cdot L^{-1}$ in the

sample was obtained, after which the sensor response slowed down. For the lowest concentrations of trypsin solution, the measuring time was about 4 min, even though 1–2 min was more than enough for the highest concentrations.

The stability of the alcohol oxidase membranes under working conditions was tested. When comparing the sensitivity of an enzymic membrane working for a day with protease in the medium to measuring alcohol directly added in the buffer measurement, we observed the same loss of the initial sensitivity (10%).

CONCLUSION

A wide variety of assay procedures have been used for the analysis of proteases. It should be noted that the various assays and substrates are interchangeable and that the assay conditions will depend on the type of protease being analyzed. The procedure developed here could permit any protease determination, even in turbid or colored samples.

REFERENCES

1. Clark, L. C., Jr. and Lyons, C. (1962), *Ann. NY Acad. Sci.* **102,** 29–45.
2. Guilbault, G. G. (1984), *Analytical Uses of Enzymes,* Marcel Dekker, New York.
3. Turner, A. P. F., Karube, I., and Wilson, G. S. (1987), *Biosensors, Fundamentals and Applications,* Oxford University Press, New York.
4. Romette, J. L. and Thomas, D. (1988), in *Methods in Enzymology,* vol. 137, Mosbach, K. ed, Academic, San Diego, CA, pp. 44–61.
5. Coughland, M. P., Kierstan, M. P. J., Border, P. M., and Turner, A. P. F. (1988), *J. Microb. Meth.* **8,** 1–50.
6. Schmid, R. D. and Scheller, F. (1989), *Biosensors, Applications in Medicine, Environmental Protection and Process Control.* GBF Monographs, vol. 13, VCH, New York.
7. Hall, E. A. H. (1990), *Biosensors,* Open University Press, Buckingham.
8. Buck, R. P., Hatfield, W. E., Umana, M., and Bowden, E. (1990), *Biosensor Technology, Fundamentals and Applications,* Marcel Dekker, New York.
9. Coulet, P. R., Bardeletti, G., and Sechaud, F. (1991), in *Bioinstrumentation and Biosensors,* Wise, D. L., ed., Marcel Dekker, New York, pp. 755–795.
10. Guilbault, G. G., Suleiman, A. A., Fatibello-Filho, O., and Nabirahni, M. A. (1991), in *Bioinstrumentation and Biosensors,* Wise, D. L., ed., Marcel Dekker, New York, pp. 659–692.
11. Blum, L. J. and Coulet, P. R. (1991), *Biosensor Principles and Applications,* Marcel Dekker, New York.
12. Nanjo, M. and Guilbault, G. G. (1975), *Anal. Chim. Acta.* **75,** 169–180.
13. Belgith, H., Romette, J. L., and Thomas, D. (1987), *Biotech. Bioeng.* **30,** 1001–1005.

14. Kitagawa, Y., Ameyama, M., Nakashima, K., Tamiya, E., and Karube, I. (1987), *Analyst* **112,** 1747–1749.
15. Hikuma, M., Kubo, T., Yasuda, T., Karube, I., and Susuki, S. (1979), *Biotech. Bioeng.* **21,** 1845–1853.
16. Blaedel, W. J. and Engstrom, R. C. (1980), *Anal. Chem.* **52,** 1691–1697.
17. Mascini, M., Memoli, A., and Olana, F. (1989), *Enzyme Microb. Technol.* **11,** 297–301.
18. Karube, I. and SangMok Chang, M. E. (1991), in *Biosensor Principles and Applications,* Blum, L. J. and Coulet, P. R., eds., Marcel Dekker, New York, pp. 267–301.
19. Albery, W. J., Bartlett, P. N., Cass, A. E. G., and Sim, K. W. (1987), *J. Electroanal. Chem.* **218,** 127–134.
20. Sim, K. W. (1990), *Biosensors Bioelectron.* **5,** 311–325.
21. Kulys, J. and Schmid, R. D. (1991), *Biosensor Bioelectronics* **6,** 43–48.
22. Guilbault, G. G., Danielson, B., Mandenius, C. F., and Mosbach, K. (1983), *Anal. Chem.* **55,** 1582–1585.
23. Zhao, J. and Buck, R. P. (1991), *Biosensors Bioelectronics* **6,** 681–687.
24. Smith, V. J., Green, R. A., and Hopkins, T. R. (1989), *J. Assoc. Off. Anal. Chem.* **72,** 30–33.
25. Peguin, S., Coulet, P. R., and Bardeletti, G. (1989), *Anal. Chim. Acta.* **222,** 83–93.
26. Bardeletti, G., Sechaud, F., and Coulet, P. R. (1991), in *Biosensor Principles and Applications,* Blum, L. J. and Coulet, P.R., eds., Marcel Dekker, New York, pp. 7–45.
27. Scheller, F. and Schubert, F. (1992), in *Biosensors,* Elsevier, Amsterdam, pp. 307–310.
28. Godfrey, T. and Reichelt, J. (1983), *Industrial Enzymology. The Application of Enzymes in Industry,* The Nature Press.
29. Bardeletti, G., Sechaud, F., and Coulet, P. R. (1986), *Anal. Chim. Acta.* **187,** 47–54.
30. Rick, W. (1981), in *Methods of Enzymatic Analysis,* vol. 2, VCH Verlagsgesellschadt mbH, Weinheim, pp. 1013–1024.
31. Monsan, P. and Combes, D. (1988), in *Methods in Enzymology,* vol. 137, Mosbach, K. ed., Academic, San Diego, CA, pp. 584–598.
32. Sahm, H. and Wagner, F. (1973), *Eur. J. Biochem.* **36,** 250–256.
33. Kato, N., Omori, Y., Tani, Y., and Gata, K. (1976), *Eur. J. Biochem.* **64,** 341–350.
34. Danielson, B. (1991), in *Biosensor Principles and Applications,* Blum, L. J., and Coulet, P. R., eds., Marcel Dekker, New York, pp. 83–105.
35. Dixon, M. and Webb, E. C. (1979), *Enzymes,* 3rd ed., Academic, New York, pp. 261–262.
36. Sarath, G., De la Motte, R., and Wagner, F. W. (1989), in *Proteolytic Enzymes—a Practical Approach,* Beynon, B. J. and Bond, J. S., eds, IRL Press, Oxford, pp. 25–55.
37. Bergmeyer, H. U., Bernt, E., Grossl, M., and Michal, G. (1981),in *Methods of Enzymatic Analysis,* VCH Verlagsgesellschadt mbH, Weinheim, pp. 308–317.