Journal of Chromatography A, 652 (1993) 225–232
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 203

Separation of amino acid–oxazole derivatives of the redox coenzyme pyrroloquinoline quinone by capillary zone electrophoresis

Yukihiro Esaka, Yasuo Yamaguchi, Kenji Kano* and Masashi Goto

Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502 (Japan)

ABSTRACT

Condensation products (oxazole derivatives) from the reaction of the coenzyme pyrroloquinoline quinone (PQQ) with several α-amino acids were successfully separated by capillary zone electrophoresis. Addition of a certain organic solvent such as dimethyl sulphoxide to the electrolyte solution is essential for reproducible and complete separation. The organic modifier appears to prevent the oxazole derivatives from adsorbing on the capillary wall. Product analysis of the condensation reactions of PQQ with amino acids was performed by this method and the mechanism is discussed briefly. PQQ-spiked bovine serum was also analysed. Unsubstituted type 1 oxazole derivative was predominantly detected. This result suggests that most of the PQQ in mammalian fluids, if any, exists as PQQ derivatives, probably as a type 1 oxazole derivative.

INTRODUCTION

Pyrroloquinoline quinone (PQQ, see Fig. 1) was discovered in bacteria as a novel redox coenzyme of non-flavin or nicotinamide-dependent dehydrogenages in 1979 [1,2]. In early studies, several enzymes such as bovine serum amine oxidase [3,4] and methylamine dehydrogenase [5,6] were reputed to contain covalently bound PQQ and PQQ derivatives. Further, the occurrence of free PQQ in mammalian fluids and also, for example, in serum and milk was proposed [7,8]. Recently, however, topa quinone [9,10] and tryptophan tryptophyl quinone [11] have been proved to be the organic cofactors of copper-containing amine oxidases and methylamine dehydrogenase, respectively. In turn, the occurrence of PQQ in mammalian fluids has been questioned, while the biological importance [12–14] and pharmaceutical activity of PQQ in mammalian [11,15] continue to be emphasized.

Such confused circumstances appear to be partly due to a lack of reliable, sensitive and selective analytical methods for PQQ. We have reported a high-performance liquid chromatographic (HPLC) determination of free PQQ with an electrochemical detector coupled with amplification by a redox-cycling reaction of PQQ with glycine [7] and applied it to the determination of free PQQ in milk and swine serum [16]. However, no free PQQ was detected. In contrast, the immediate disappearance of free PQQ added to those biological fluids has been proved. This suggests that most of the PQQ in such biological media, if any, would be converted into some redox-inactive derivatives. Hence there seems to be an urgent need to develop an analytical method to speciate PQQ and its derivatives in biological fluids in order to provide some breakthrough in this confused field.

PQQ is known to be very reactive towards nucleophilic reagents [17–19]. Amino acids can be considered to be the most important and

* Corresponding author.

0021-9673/93/$06.00

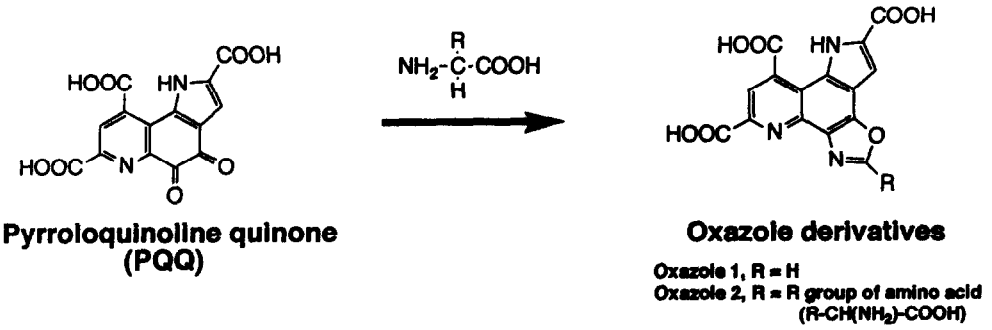

Fig. 1. Formation of amino acid–oxazole derivatives of PQQ.

widely distributed compounds among the many nucleophilic compound in biological fluids in view of the nucleophilic reaction towards PQQ. As shown in Fig. 1, free PQQ reacts readily with amino acids under aerobic conditions to yield corresponding oxazole derivatives as the final products [19–22]. The oxazole derivatives show variety in the structure of the R groups. Following Van Kleef *et al.* [21], the oxazoles with a hydrogen atom and amino acid residues as the R group will be called oxazoles 1 and 2, respectively, in this paper. All these oxazole derivatives have no redox–catalytic activity. Our hypothesis here is that the oxazole derivative(s) is (are) the major product(s) from PQQ in reactions in biological fluids.

Although reversed-phase (RP) HPLC with gradient elution [21] and ion-exchange chromatography [22] have been reported for the determination of the oxazoles, they might not necessarily be suitable for the separation of oxazole derivatives. The reasons are that the oxazole derivatives are strongly charged molecules owing to the three carboxyl groups and the acidic and basic amino acid residues and that their structures are only slightly different from each other (Fig. 1). RP-HPLC would not be suitable for the separation of such charged molecules and structurally related compounds with an identical charge might not be separated completely by ion-exchange chromatography.

Capillary zone electrophoresis (CZE) has a very high resolution for charged samples. Our aims in this work were to separate all oxazole derivatives by employing CZE with good resolution and to apply the method to the product analysis of the reaction between PQQ and each amino acid. Further, the speciation of PQQ in biological fluids will be discussed based on analyses of PQQ-spiked bovine serum samples.

EXPERIMENTAL

Materials

PQQ was obtained from Ube Industry (Tokyo, Japan) and used as received. Bovine serum was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical-reagent grade.

Apparatus

Electrophoretic separation was performed in a fused-silica tube (GL Science, Tokyo, Japan) of 0.05 mm I.D. and a length of 550 mm, with an effective length for separation, *i.e.*, the length from the injection end to the detection cell, of 300 mm unless noted otherwise. A Matsusada Precision Devices (Kusatu, Japan) Model HCZE-30PNO high-voltage d.c. power supply, which can deliver high voltages up to 30 kV, was employed to supply the potential across the capillary. For UV–Vis spectrophotometric detection, a Jasco (Tokyo, Japan) 875-UV detector was employed. The detection wavelength was set at 250 or 420 nm. The flow cell was modified for capillary electrophoresis in our laboratory. A Shimadzu (Kyoto, Japan) Chromatopac C-R6A was used for data processing.

Sample preparation

Oxazole derivatives were generated by incubation of PQQ (0.01 *M*) and each amino acid

(0.004 g/ml) in 200 m*M* acetate buffer (pH 3.5) or 100 m*M* phosphate buffer (pH 7.9) at a volume ratio of 1:9. The incubation period was about 2 days at a room temperature. Oxazole 1 from glycine and oxazole 2 from valine were purified by thin-layer chromatography with silica gel plates (Kieselgel 60 F_{254}; Merck) in a mixed solvent of methanol–chloroform–water (70:30:7). These were used as the standard samples. In routine analysis, the reaction mixtures were subjected to CZE analysis without any purification.

A bovine serum sample was pretreated as follows. The sample was applied to an open column packed with DEAE-cellulose and washed with 200 ml of 0.1 *M* phosphate–Tris buffer (pH 6.0). The resin at the top of the column was removed and suspended in 30 ml of 0.5 *M* NaCl solution. After adjusting the pH of the solution to 0.5 with HCl to suppress the acid dissociation, adsorbed compounds were removed from the resin by shaking for 15 min and then extracted with isobutyl alcohol. The extracted solution was concentrated by evaporation. Control experiments for the standard oxazole 1 added to bovine serum showed a recovery of more than 95%.

Procedure

Just before each run, a separation capillary tube was purged with 0.1 *M* NaOH solution for 2 min and then with a desired buffer for 3 min by using an aspirator. The two ends of the tube were then dipped into two separate 1.5-ml reservoirs filled with the same buffer. The end at which samples were introduced was connected with a platinum electrode to positive high voltage, while the other end was connected with a platinum electrode to ground. Samples were introduced by siphoning at a height of 15 cm for a 5–10-s period.

RESULTS AND DISCUSSION

CZE separation of oxazole derivatives

In preliminary experiments in which electrophoresis of the standard samples (oxazole 1 from glycine and oxazole 2 from valine) was carried out with phosphate buffer containing no modifier, the oxazoles did not exhibit a peak or, even if a peak was detected, the electropherograms resulted in very poor reproducibility. We ascribe this phenomenon to the adsorption of the analytes on the capillary wall. To overcome such an adsorption effect, we examined several organic solvents as modifiers [23]. In this work, 10 m*M* phosphate buffer (pH 8.0) containing 5 or 10% (v/v) of a certain organic solvent was employed as an electrolyte solution, in which the three carboxyl groups in the oxazole derivatives are expected to be acid dissociated, as in the case of PQQ [24,25]. The addition of any one of dimethyl sulphoxide (DMSO), methanol (MeOH) and acetonitrile (ACN) was found to be efficient for obtaining reproducible peaks of the oxazoles. From the point of view of the complete separation of the oxazole derivatives (see below), however, DMSO was most preferred as a modifier for high resolution. The theoretical plate number (N) was calculated to be 2.75×10^5 for oxazole 1 based on the equation $N = l^2/2Dt$, where l, D and t are the separation distance, the diffusion coefficient and the migration time of a sample, respectively [26]. In the above calculation, the D value of PQQ ($3.9 \cdot 10^{-6}$ cm^2 s^{-1} [24]) was used. When MeOH was used as a modifier, a somewhat lower resolution was obtained with DMSO, and ACN gave an even lower resolution. This might be explained in terms of the relatively small value of D in the DMSO mixed solvent owing to its high viscosity and/or its large number of solvating molecules compared with the others. The electroosmotic flow-rate was virtually independent of the organic solvents used at such low concentrations, although at concentrations above 20% a marked effect on the flow-rate was observed, as has been reported elsewhere [27].

Reactions of PQQ with amino acids

Product analyses of the reactions of PQQ with several amino acids were performed by the present method together with absorption spectroscopy. Peak identification was performed by addition of standard samples and comparison of two electropherograms detected at 250 and 420 nm. Oxazoles are reported to exhibit a characteristic adsorption band around 420 nm [21], at

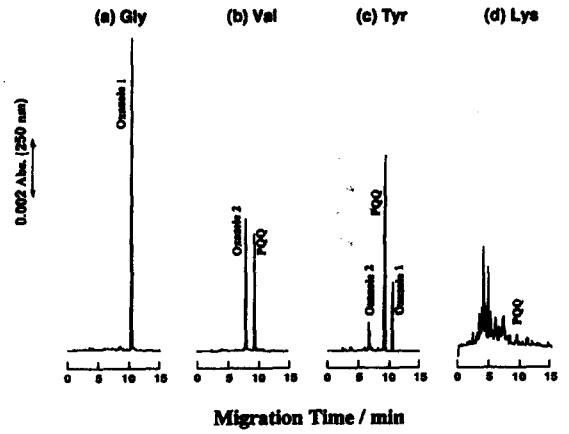

Fig. 2. Electropherograms of reaction solutions of PQQ with (a) glycine, (b) valine, (c) tyrosine and (d) lysine at pH 7.9 for 48 h at room temperature. Electrolyte solution, 10 m*M* phosphate buffer (pH 8.0) containing 5% (v/v) DMSO; capillary, 550 mm × 0.05 mm I.D. (300 mm effective length); separation voltage, 23 kV; current, 17 μA; injection (siphoning), 15 cm, 7 s; detection wavelength, 250 nm.

which the absorption of PQQ and other adducts is very weak compared with that at 250 nm.

Fig. 2 shows electropherograms of the reaction mixtures (pH 7.9) of four amino acids detected at 250 nm and Table I summarizes the yields of oxazoles 1 and 2 generated from several amino acids. Although the reaction products depend greatly on the amino acids and the reaction pH, we divided the amino acids into five classes (see Table I and below).

A typical amino acid in class 1 is glycine, with which the reaction led to the formation of oxazole 1 exclusively (Fig. 2a). The absorption spectrum of the reaction mixture gave a characteristic absorption maximum of oxazole 1 around 420 nm (Fig. 3, curve 1). Virtually identical results were obtained at pH 3.5 and 7.9. A similar behaviour was observed for the reaction with tryptophan (Table I). These results are in close agreement with those of other workers [20–22]. Threonine can be included in this class, although oxazole 2 was detected as a minor product.

In the reaction with valine, oxazole 2 was predominantly generated, while some PQQ remained unchanged (Fig. 2b). The absorption spectrum of the reaction mixture exhibited an

TABLE I

YIELDS OF OXAZOLES 1 AND 2 IN THE REACTIONS OF PQQ WITH SEVERAL AMINO ACIDS AT pH 3.5 AND 7.9

Incubation: 48 h at room temperature. Lys and Arg (class IV) yielded no detectable oxazoles but some adducts different from oxazoles. Pro, Hyp, Cys and Cys-Cys (class V) yielded no adducts.

Amino acid	Yield (%)				Class
	pH 3.5		pH 7.9		
	Oxazole 1	Oxazole 2	Oxazole 1	Oxazole 2	
Gly	100	–	100	–	I
Trp	97	–	92	–	I
Thr	75	22	84	12	I
Val	–	81	<1	67	II
Ala	3	87	13	72	II
Leu	–	93	6	72	II
Ile	–	84	3	61	II
Phe	3	97	2	83	II
Asn	–	95	6	70	II
Gln	–	97	3	90	II
Asp	–	85	–	38	II
Glu	4	92	6	50	II
Met	–	68	–	32	II
His	–	94	–	27	II
Tyr	–	73	23	12	III
Ser	2	94	91	8	III

absorption band around 420 nm (Fig. 3, curve 2). The product was virtually independent of the reaction pH. This class (class II) includes alanine, leucine, isoleucine, phenylalanine, asparagine, glutamine, aspartic acid and glutamic acid (Table I). For some of these amino acids, oxazole 1 was also formed as a minor product. Methionine and histidine yielded the corresponding oxazoles 2 as major products at pH 3.5 and 7.9 and can therefore be assigned to class II. However, there were several minor products different from the oxazoles.

With tyrosine, both oxazoles 1 and 2 were detected (Fig. 2c), but the ratio of oxazole 1 to oxazole 2 depended greatly on the reaction pH: at pH 3.5 oxazole 2 was generated preferentially, whereas at pH 7.9 oxazole 1 was the major product. A very similar behaviour was observed for serine. Such a pH-dependent reaction of

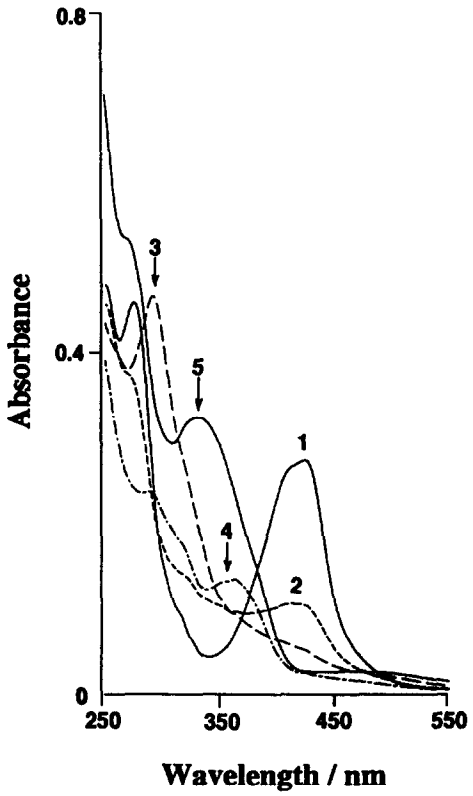

Fig. 3. Absorption spectra of reaction solutions of PQQ with (1) glycine, (2) valine, (3) lysine and (4) arginine and (5) free PQQ. Reaction conditions: PQQ (0.01 *M*)–amino acid (0.004 g/ml in 100 m*M* phosphate buffer, pH 7.9) (1:9) incubated for 48 h at room temperature. Before measurement, reaction solutions were diluted with a fivefold volume of 10 m*M* phosphate buffer (pH 7.9).

serine was reported first by Adachi [28] and then in detail by Itoh *et al.* [22].

Amino acids that did not yield any detectable oxazoles were assigned to class IV. Lysine exhibited a very complicated electropherogram (Fig. 2d), indicating the existence of several reaction pathways. The absorption spectrum of the reaction mixture was completely different from that of glycine. Although the formation of a small amount of oxazole derivatives is suggested by a shoulder around 420 nm (Fig. 3, curve 3), there appeared to be no significant peak in an electropherogram detected at 420 nm (not shown). The reaction with arginine resulted in the formation of one major adduct. This adduct, however, seems to be different from the oxazoles, because detection at 420 nm showed only a small peak and the absorption spectrum of the mixture was completely different from that of glycine (Fig. 3, curve 4).

Class V includes proline, hydroxyproline, cysteine and cystine. These amino acids did not give adducts, although cysteine is oxidized to cystine by PQQ [29].

The results suggest that there are two major competing reaction pathways to give oxazoles 1 and 2. We propose the mechanism shown in Fig. 4 for the formation of oxazoles. The reaction between PQQ and amino acids has been reported to proceed via an ionic mechanism involving carbinolamine-type intermediates which are dehydrated to give iminoquinones [9,28]. When C_α–C_β bond cleavage followed by decarboxylation proceeds from the iminoquinones, oxazole 1 is generated as a major product. The pathway is similar to that proposed by Van Kleef *et al.* [21]. In contrast, when decarboxylation is facilitated in the iminoquinone prior to the C_α–C_β bond fission, oxazole 2 will be predominantly produced. This reaction pathway has already been proposed by Ohshiro and co-workers [19,22].

The predominant path seems to depend on the amino acid residues and also the reaction conditions. For the amino acids in class I (except glycine), C_α–C_β bond cleavage would be facilitated, but decarboxylation is the path for the amino acids in class II. With serine and tyrosine (class III), base-catalysed C_α–C_β bond fission would occur rather than the decarboxylation at an elevated pH [22]. Lysine and arginine in class IV and histidine in class II are basic in nature and converted into some adducts different from oxazoles in major or minor processes. These amino acids have two nucleophilic groups in each molecule. In view of this, it is interesting to refer to the reactions of ethylenediamine [30] and aminoguanidine [31] with PQQ, in which pyrazine and triazine derivatives, respectively, are generated.

Fig. 5 illustrates an electropherogram of a mixture of the reaction solutions of PQQ with most of the amino acids in classes I, II and III (glycine, tryptophan, threonine, valine, alanine, leucine, phenylalanine, asparagine, glutamine,

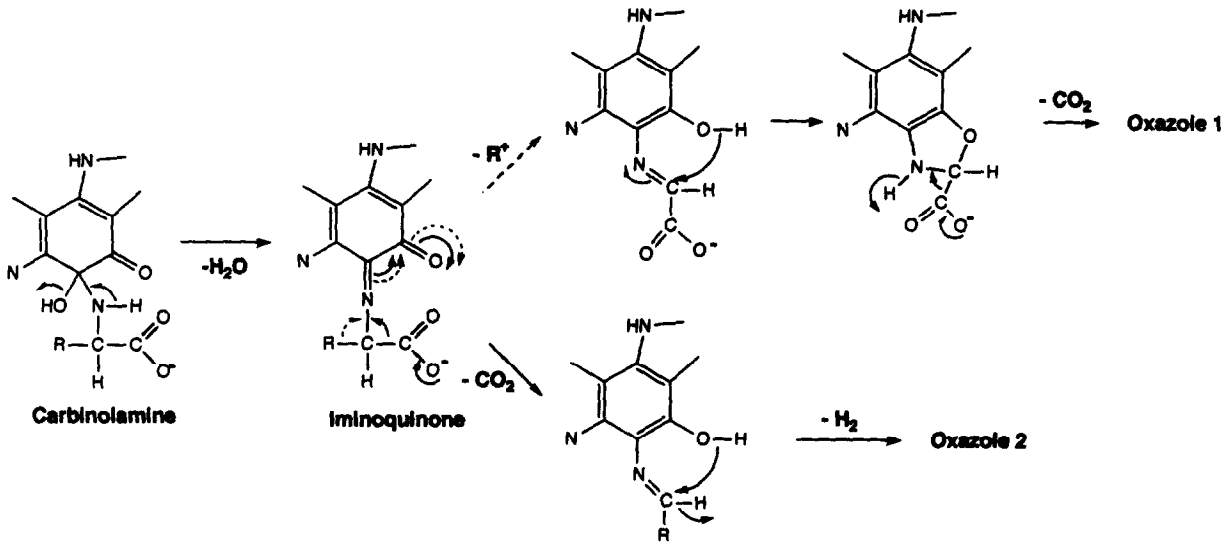

Fig. 4. Proposed mechanism of the formation of oxazoles 1 and 2.

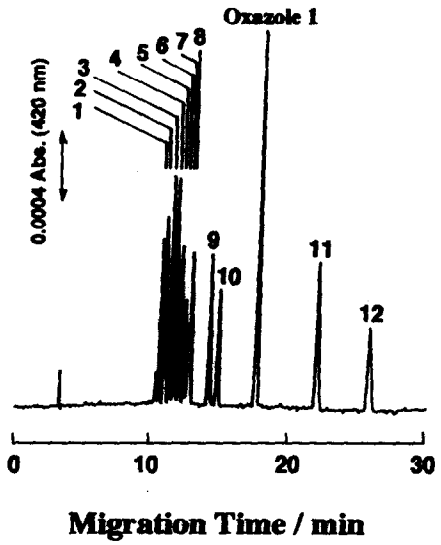

Fig. 5. Electropherogram of a mixture of reaction solutions of PQQ with several amino acids at pH 3.5. Electrolyte solution, 10 m*M* phosphate buffer (pH 8.0) containing 5% (v/v) DMSO; capillary, 700 mm × 0.05 mm I.D. (450 mm effective length); separation voltage, 25 kV; current, 14 μA; injection (siphoning), 15 cm, 7 s; detection wavelength, 420 nm. Peaks 1–12: oxazoles 2 generated from tyrosine, histidine, phenylalanine, leucine, glutamine, asparagine, threonine, valine, serine, alanine, glutamic acid and aspartic acid, in that order.

aspartic acid, glutamic acid, histidine, tyrosine and serine) except isoleucine and methionine. Each peak was identified by the addition of the corresponding oxazole derivative. The oxazole 2 from isoleucine gave a peak at a position identical with that of the oxazole 2 from leucine owing to their strong structural similarity. The peak of the oxazole 2 from methionine overlapped with that of glutamine. The amino acids in class IV would make the electropherograms complicated because they yield many kinds of minor products, as mentioned above.

Baseline resolution of most of the oxazole derivatives was obtained, except for two oxazoles 2 from threonine and valine, within *ca.* 25 min. The present separation appears to be much superior to those obtained with HPLC in terms of the resolution and the separation time. The detection limit for oxazole 1 was *ca.* 50 fmol ($S/N = 5$, 10 μ*M*, *ca.* 5-nl injection).

The detailed mechanism of the reaction of PQQ with amino acids has not been completely elucidated. However, this work demonstrates that CZE is a powerful tool for such mechanistic and/or kinetic studies. Further research is in progress.

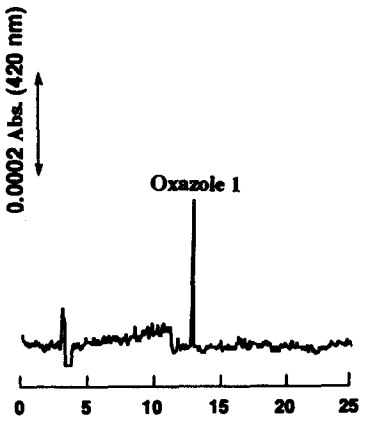

Fig. 6. Electropherogram of PQQ-spiked bovine serum sample. Electrolyte solution, 10 m*M* phosphate buffer (pH 8.0) containing 5% (v/v) DMSO; capillary, 550 mm × 0.05 mm I.D. (300 mm effective length); separation voltage, 17 kV; current, 13 μA, injection (siphoning), 15 cm, 7 s; detection wavelength, 250 nm. See text for pretreatment of the sample.

Analysis of PQQ-spiked bovine serum and native bovine serum

PQQ and its oxazole derivatives in PQQ-spiked bovine serum were determined using the present CZE method. The pretreatment was carried out about 48 h after the PQQ addition. An electropherogram of PQQ-spiked bovine serum sample is given in Fig. 6. For this sample, the PQQ concentration added to serum was $5 \cdot 10^{-6}$ *M* and a 30-fold concentration was carried out in the preparation. Oxazole 1 was observed as a major peak component. The peak of oxazole was identified by adding the standard oxazole 1 to the pretreated serum sample. The spectrum of the PQQ-spiked sample also supported the oxazole formation (not shown). When PQQ was added at a higher concentration of $2 \cdot 10^{-4}$ *M*, some oxazoles 2 together with the unreacted free PQQ were detected as minor peaks.

These results indicate that most of the PQQ existing in serum in the oxidized form is converted into oxazole 1 during the course of the reaction with free amino acids. The reason why oxazole 1 is a major product seems to be that the rate of the formation of oxazole 1 from glycine is much faster than that of oxazole 2 from other amino acids.

For a native (non-PQQ-spiked) bovine serum sample, however, no oxazole derivatives were found, even though a *ca.* 100-fold concentration was performed in the pretreatment. Hence the concentration of PQQ–oxazole derivatives in the bovine serum might be expected to be less than 10^{-7} *M*, if present at all. For further studies, some method of more effective and selective preconcentration seems to be required.

REFERENCES

1 J.A. Duine, J. Frank, Jr. and J.K. van Zeeland, *FEBS. Lett.*, 108 (1979) 443.
2 S.A. Salisbury, H.S. Forret, W.B.T. Cruse and O. Kennard, *Nature*, 280 (1979) 843.
3 C.L. Lobenstein-Verbeek, J.A. Jongejan, J. Frank and J.A. Duine, *FEBS Lett.*, 170 (1984) 305.
4 M. Ameyama, M. Hayashi, K. Matsushita, E. Shinagawa and O. Adachi, *Agric. Biol. Chem.*, 48 (1984) 561.
5 W.S. McIntire and J.T. Stults, *Biochem. Biophys. Res. Commun.*, 141 (1986) 562.
6 F.M.D. Vellieux and W.G. Hol, *FEBS Lett.*, 255 (1989) 460.
7 R. Flückiger, T. Woodtli and P.M. Gallop, *Biochem. Biophys. Res. Commun.*, 153 (1988) 353.
8 J.A. Jongejan and J.A. Duine (Editors), *PQQ and Quinoproteins*, Kluwer, Dordrecht, 1989, pp. 111–143.
9 S.M. Janes, D. Mu, D. Wemmer, A.J. Smith, S. Kaur, D. Maltby, A.J. Burlingame and J.P. Klinman, *Science*, 248 (1990) 981.
10 D.E. Brown, M.A. McGuirl, D.M. Dooley, S.M. Janes, D. Mu and J.P. Klinman, *J. Biol. Chem.*, 266 (1991) 4049.
11 W.S. McIntire, D.E. Wemmer, A. Chistoserdov and M.E. Lidstrom, *Science*, 252 (1991) 817.
12 P.M. Gallop, M.A. Paz, R. Flückiger and E. Henson, *2nd International Symposium on PQQ and Quinoproteins, November 1991, Yamaguchi, Japan*, Abstracts, p. 40.
13 J. Killgore, C. Smidt, L. Duich, N. Romero-Chapman, D. Tinker, K. Reiser, M. Merko, D. Hyde and R.B. Rucker, *Science*, 245 (1989) 850.
14 M. Ameyama, K. Matsushita, E. Shinagawa and O. Adachi, *Vitam. Horm.*, 46 (1991) 229.
15 H. Nishigori, *Nippon Nogeikagaku Kaishi*, 66 (1992) 1657 and references cited therein.
16 Y. Esaka, K. Kano, M. Sukeguchi and M. Goto, *Anal. Sci.*, 9 (1993) 207.
17 J.A. Duine, J. Frank, Jr. and J.A. Jongejan, *Adv. Enzymol.*, 59 (1987) 170–212; and references cited therein.
18 J.A. Jongejan and J.A. Duine (Editors), *PQQ and Quinoproteins*, Kluwer, 1989, pp. 195–241.

19 S. Itoh and Y. Ohshiro, *Bioorg. Chem.*, 19 (1991) 169, and references cited therein.
20 D.R. Sleath, J.B. Noar, G.A. Eberlein and T.C. Burice, *J. Am. Chem. Soc.*, 107 (1985) 3328.
21 M.A.G. van Kleef, J.A. Jongejan and J.A. Duine, *Eur. J. Biochem.*, 183 (1989) 41.
22 S. Ihoh, M. Mure, A. Suzuki, H. Murao and Y. Ohshiro, *J. Chem. Soc., Perkin Trans. 2* (1992) 1245.
23 N.J. Reinhoud, U.R. Tjaden, H. Irth and J. van der Greef, *J. Chromatogr.*, 574 (1992) 327.
24 K. Kano, K. Mori, B. Uno, T. Kubota, T. Ikeda and M. Senda, *Bioelectrochem. Bioenerg.*, 23 (1990) 227.
25 K. Kano, K. Mori, B. Uno, T. Kubota, T. Ikeda and M. Senda, *Bioelectrochem. Bioenerg.*, 24 (1990) 193.
26 J.W. Jorgenson and K.D. Lukacs, *Anal. Chem.*, 53 (1981) 1298.
27 C. Schwer and E. Kennder, *Anal. Chem.*, 63 (1991) 1801.
28 O. Adachi, *Nippon Nogeikagaku Kaishi*, 66 (1992) 1665, and references cited therein.
29 S. Itoh, N. Kato, M. Mure and Y. Ohshiro, *Bull. Chem. Soc. Jpn.*, 60 (1987) 420.
30 M. Mure, S. Itoh and Y. Ohshiro, *Chem. Lett.*, (1989) 1491.
31 M. Mure, K. Nii, S. Itoh and Y. Ohshiro, *Bull. Chem. Soc. Jpn.*, 63 (1990) 417.