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Following the Lipase Catalyzed Enantioselective Hydrolysis of Amino Acid Esters with Capillary Electrophoresis Using Contactless Conductivity Detection

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ABSTRACT The progress of the enzymatic hydrolysis of racemic mixtures of the enantiomers of the methyl esters of serine and threonine was monitored. This was possible in a reaction vessel of 1.5 mL by direct sampling of volumes in the nanoliter-range directly into an electrophoresis capillary. Contactless conductivity detection was used for quantification as the analytes are not accessible by UV-detection in capillary electrophoresis. Porcine pancreatic lipase and wheat germ lipase both showed a preference for the L-enantiomers of both amino acid esters. The selectivity of the porcine lipase between the two L-esters of the two amino acids was also studied and it was found that the production of L-threonine had priority over L-serine. *Chirality 22:331–335, 2010.*

KEY WORDS: enzymatic reactions; enantiomeric separations; ester hydrolysis; lipase kinetic resolution; amino acids; capillary electrophoresis; conductivity detection

INTRODUCTION

Capillary electrophoresis (CE) is an attractive method for the separation of enantiomers as in contrast to HPLC it is not necessary to use a chiral column. Chiral reagents can be dissolved in the relatively low volumes of the separation buffer and do not have to be covalently bound to the packing material of separation columns. Optimization is possible by adjusting concentration, pH or ratios of different dissolved reagents without having to resort to modifications of a stationary phase. This application has therefore become one of the major uses of CE. For a recent review, see for example Ref 1. A further advantage of CE is the fact that only small sample volumes are required as the separation is carried out in narrow capillaries.

A shortcoming of CE has long been the limitation of commercial instrumentation to UV-absorbance or fluorescence detection. Because of the short optical pathlengths available, this has meant that weakly absorbing species, such as most amino acids, could only be detected following chemical derivatization to render them accessible by optical means. Often studies of amino acids by CE have been restricted to those few species which have an aromatic moiety and are thus easily detectable by UV-absorbance. See for example, the recent review by Lindner and coworkers.[2] This shortcoming can, however, be overcome by using conductivity detection, which is universal for all ionic species.

Conductivity detection has been used for electromigration separation techniques for a long time, but has played a marginal role until the introduction of the contactless approach relying on a pair of external tubular electrodes.[3,4] The method has been termed C^4D for "capacitively coupled contactless conductivity detection." Its fundamentals have been studied extensively[5–8] and at least two commercial devices are now available and can be retrofitted to existing CE-instrumentation. Recent reviews on applications of C^4D are also available.[9–11]

Several research groups have demonstrated the determination of amino acids at low pH in cationic form using contactless conductivity detection.[12–16] Gong et al.[17] demonstrated that the determination of the enantiomers of non-UV-absorbing amino acids is possible by CE-C^4D using a combination of the noncharged additives hydroxypropyl-β-cyclodextrin and (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid ($18C6H_4$) as the chiral separators. It has furthermore been shown that capillary electrophoresis-capacitively coupled contactless conductivity detection (CE-C^4D) may be used for the monitoring of enzymatic reactions when the species involved are not detectable by optical means. The nonionic species glucose, ethanol, ethyl acetate, and ethyl butyrate were made accessible for analysis by CE via charged products or byproducts obtained in enzymatic conversions using hexokinase,

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glucose oxidase, alcohol dehydrogenase, and esterase, respectively.[18] Recently, urea was also determined by CE-C^4D in clinical samples via enzymatic conversion to ammonium ion with urease.[19]

The application of CE-C^4D to the monitoring of the lipase-catalyzed hydrolysis of amino acid esters is reported herein. Many workers have investigated the use of lipases for enantioselective synthesis. In particular Reetz et al.[20–22] have studied the optimization of the enzyme structures in an approach named "directed evolution." This reaction has also been investigated as a useful path to the resolution of racemates of the amino acids, and work in this area was reviewed by Miyazawa.[23] More recently, Malhotra and Zhao[24] have researched the enantioseparation of the esters of α-amino acids by lipase in an ionic liquid.

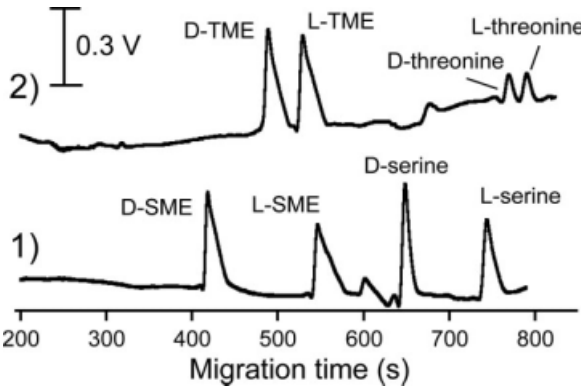

Fig. 1. Electropherograms of the concurrent separation of the enantiomers of (1) DL-SME and DL-serine, and (2) DL-TME and DL-threonine. Buffer: 2 M acetic acid/5 mM $18C6H_4$. Capillary: 50 μm i.d., total length 50 cm, and effective length 45 cm. Injection: hydrodynamic, 15 cm height elevation for 10 s. Separation voltage: +15 kV.

MATERIALS AND METHODS

Chemicals and Procedures

All chemicals were of reagent grade and deionized water (Millipore, Bedford, MA) was used throughout. All chemicals were purchased either from Sigma, Fluka, Aldrich (Buchs, Switzerland), or Acros Organics (Geel, Belgium). The details of the suppliers are as follows: DL-serine methyl ester hydrochloride (Sigma), L-serine methyl ester (SME) hydrochloride (Aldrich), DL-threonine methyl ester (TME) hydrochloride (Sigma), L-threonine methyl ester hydrochloride (Fluka), DL-serine (Fluka), L-serine (Sigma), DL-threonine (Fluka), and L-threonine (Sigma). Acetic acid was purchased from Fluka. HPLC-grade acetonitrile was obtained from Acros. Sodium bicarbonate was purchased from Sigma. (+)-(18-(Crown-6)-2,3,11,12-tetracarboxylic acid was bought from Fluka. The enzymes lipase from porcine pancreas (Type II) and lipase from wheat germ (WGL) (Type I) were also purchased from Sigma. All stock solutions were prepared in deionized water. Fused-silica capillaries of 50 μm ID and 375 μm OD were purchased from Polymicro Technologies (Phoenix, AZ) and were preconditioned with 1 mM sodium hydroxide solution and then flushed with water followed by 1 mM hydrochloride solution and again by water. Before use, they were flushed with the running buffer. All capillaries had a total length of 50 cm and 45 cm effective length. Sample injection was carried out manually directly from the reaction vessels in a hydrodynamic manner at 10 cm elevation for 10 s. The separation voltage was +15 kV unless stated otherwise.

Instrumentation

Separations were carried out on an electrophoretic instrument made in-house which is based on a high-voltage power supply from Spellman (model CZE 2000, Pulborough, UK). It comprises a Perspex box which is fitted with a microswitch to interrupt the power supply for safety reasons when opening. The contactless conductivity detector was also constructed in-house, and details can be found elsewhere.[25,26] The cell design detailed in Zhang et al.[27] was used. Data acquisition was carried out with a Macintosh personal computer (Apple, Cupertino, CA) using a MacLab/4e analog-to-digital converter system (AD Instruments, Castle Hill, Australia).

RESULTS AND DISCUSSION

Separation and Detection of the Enantiomers of Serine and Threonine

To monitor the hydrolysis, it is necessary to be able to separate and detect both enantiomers of the amino acids. DL-serine methyl ester and DL-threonine methyl ester were chosen as model compounds. These compounds do not contain benzyl groups and it would thus not be possible to detect them optically in CE. The choices were made partly also owing to the commercial availability of the esters. An electrolyte solution consisting of 2 M acetic acid to render the analytes in the protonated positively charged form and 5 mM of the chiral crown ether $18C6H_4$ [(+)-(18-(crown-6)-2,3,11,12-tetracarboxylic acid] was used as selector. This was a variation of a buffer found suitable by Gong et al.[17] for the separation of the enantiomers of amino acids.

As illustrated by electropherogram 1 of Figure 1, which is the result of the injection of a mixture of 5 mM DL-SME and 5 mM DL-serine, the enantiomers of serine can be separated from the substrate, and the two enantiomers are well resolved. Furthermore, it is possible to also separate the enantiomers of the methyl esters using the electrolyte solution containing the chiral reagent. For the enantiomers of serine a high resolution, R_s, of 3.7 was obtained (the separation factor, α, is 1.2). The enantiomeric separation of DL-SME is also good with a resolution of 3.8 ($\alpha = 1.3$). The result of the separation of a mixture of DL-TME and DL-threonine is shown in electropherogram 2 of Figure 1. The separation is generally not as good as that of the serine species, but baseline resolution was still obtained for all compounds. The R_s values were calculated as 2.0 and 1.3 for the threonine and TME enantiomers respectively ($\alpha = 1.1, 1.2$). A calibration curve for L-serine was acquired for the concentration range from 0.5 to 25 mM. The following regression equation was obtained: peak area (mV s) $= -0.0616 + 0.9451 \times$ concentration (correlation coefficient, $r = 0.9999$). A calibration curve for L-threo-

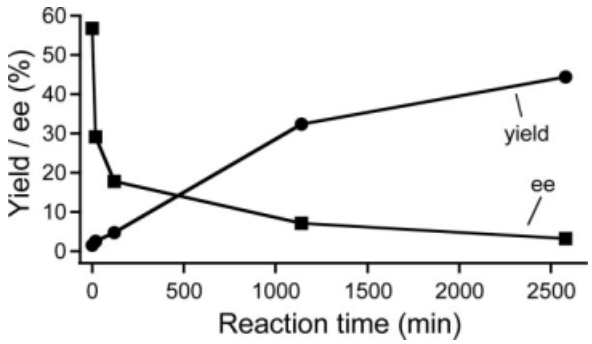

Fig. 2. Plot of the ee (■) of DL-serine and the yield (●) of L-serine resulting from the hydrolysis of DL-SME to DL-serine by PPL versus reaction time. Conditions for the reaction are given in the text. Separation as for Figure 1.

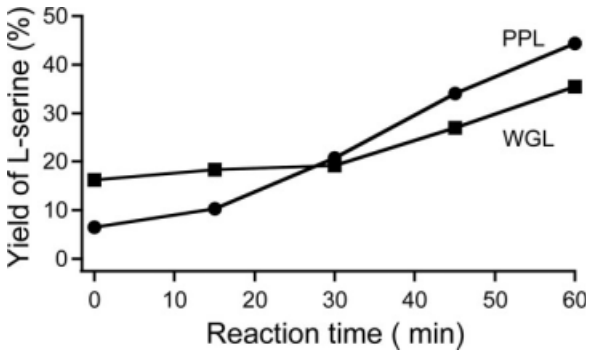

Fig. 4. Plot of the yield of L-serine produced from the hydrolysis of DL-SME by WGL (■) and PPL (●) versus reaction time. Conditions for the reaction are given in the text. Separation as for Figure 1.

nine in the range from 0.5 to 10 mM resulted in the following regression equation: peak area (mV s) = 0.012 + 0.8054 × concentration (r = 0.9993).

Hydrolysis of the Methyl Esters of DL-Serine with Porcine Lipase

The enzyme catalyzed hydrolysis requires a pH-value around 7.4. The reaction could thus not be carried out in the electrolyte solution used for separation, which has to have a low pH-value to render the analytes in the protonated cationic form. Into a 1.5 mL microvial, total volume 1 mL of a $NaHCO_3$ aqueous buffer solution (0.2 M, pH 7.8) was placed, 10 mg of a racemic mixture of DL-SMT hydrochloride was added as solid, and the enzyme porcine pancreas lipase (PPL) (2.5 mg) was added dissolved in 0.5 mL of 15% acetonitrile in water. After mixing, the vial was placed in a water bath of 37°C and the reaction was monitored for 2 days. Analysis of the reaction mixture was performed immediately after mixing, and after 20 min, 2 h, 19 h, and 43 h. The separation was carried out in the acetic acid electrolyte containing the chiral crown ether as described earlier. The concentrations of D- and L-serine were then determined from the calibration curve. Plots of enantioselectivity expressed as the enantiomeric excess (ee) value and the yield for L-serine (fraction of the L-SMT converted to L-serine) against time calculated from these values are given in Figure 2. Clearly, at the beginning of the hydrolysis, a high selectivity for L-serine was obtained, which then decreased with time as the yield increased.

Hydrolysis of the Methyl Esters of DL-Threonine with Porcine Lipase

Five milligrams of a racemic mixture of DL-TME hydrochloride and 2.5 mg of the PPL were mixed in the same fashion as described earlier for DL-SME and the mixture was incubated again at 37°C. The reaction mixture was analyzed immediately after mixing, and then after 15 min, 2 h, 5 h, and 21 h. The results are shown in Figure 3. The pattern is similar to that obtained with serine, with the most pronounced enantiomeric excess obtained early in the reaction. However, the selectivity of this reaction was generally less pronounced than for the hydrolysis of the serine ester.

Comparison Between Porcine Lipase and Wheat Germ Lipase

Next, a comparative study of the selectivity of two different lipases was carried out using the hydrolysis of DL-SMT. A total of 2.5 mg of porcine lipase and of the same amount of wheat germ lipase were added respectively to two batches of 10 mg of the racemic mixture of the substrate. The same protocol as outlined earlier was used. As

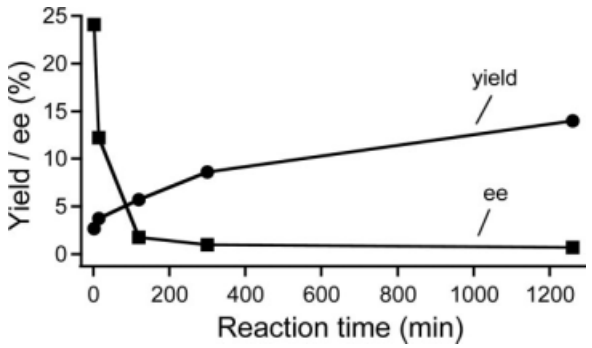

Fig. 3. Plot of the ee (■) of DL-threonine and the yield (●) of L-threonine resulting from the hydrolysis of DL-TME to DL-threonine by PPL versus reaction time. Conditions for the reaction are given in the text. Separation as for Figure 1.

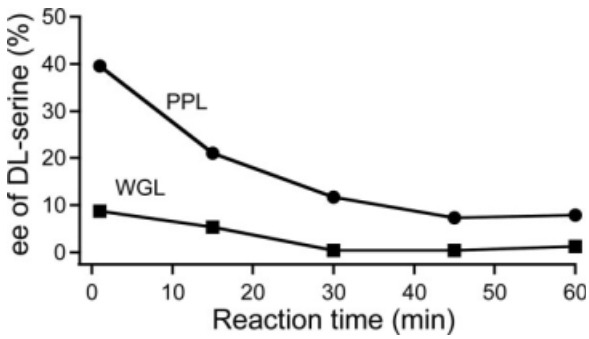

Fig. 5. Plot of the ee of DL-serine produced from the hydrolysis of DL-SME by WGL (■) and PPL (●) versus reaction time. Conditions for the reaction are given in the text. Separation as for Figure 1.

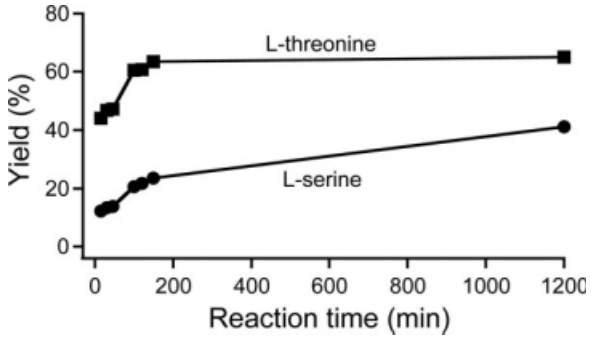

Fig. 6. Plot of the yields of L-threonine and L-serine resulting from the competitive hydrolysis between L-TME (■) and L-SME (●) by PPL versus reaction time. Conditions for the reaction are given in the text. Separation as for Figure 1.

in the previous experiments, the most pronounced enantiomeric selectivity was obtained at early times, the analysis of the reaction mixture was carried out at short intervals of 15 min for the first hour of the reaction. In Figure 4, the yield of the reaction is shown in terms of production of L-serine. It is evident that for the conditions used, the rate of reaction is slightly higher for the porcine lipase than for the wheat germ lipase. The results for the enantiomeric excess given in Figure 5 indicate that while the wheat germ lipase also prefers the L-serine ester, a higher selectivity for the L-form is found for the porcine lipase. No attempt was made to correct the data for differences in activity, but it appears that the difference is partially due to the initially higher rate of reaction for the wheat germ lipase (see Fig. 4).

The Selectivity Between the Methyl Esters of L-Serine and L-Threonine

In the final experiment, L-SME and L-TME were selected to react together to investigate the preference of the porcine lipase for the two substrates. Five milligrams of the L-SME and 5 mg of the L-TME were mixed with 2.5 mg of the PPL, using the protocol used thus far. The progress of hydrolysis was monitored over the period of 24 h and the results in terms of the yield for the two amino acids are given in Figure 6. Clearly, in the competitive situation the rate of reaction is higher for the threonine ester, the compound for which the discrimination between the two enantiomers is less pronounced. As the structure of the two amino acids is identical except for the methyl group in the β-position, the presence of the latter must be responsible for the differences observed.

CONCLUSIONS

It has been demonstrated that the CE-C^4D method is well suited for the monitoring of an enantioselective reaction involving compounds which are not readily detectable by UV-absorption measurement. The fact that only low volumes are consumed for each analysis enabled the repeated sampling from a very small reaction volume to observe the progress of the conversion, which would not have been possible by HPLC.

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