Polarimetric Assay for the Medium-Throughput Determination of α-Amino Acid Racemase Activity

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A polarimetric assay has been developed for the identification of α-amino acid racemase activity. The setup consists of a microcuvette polarimeter (40 μL volume) connected to a pipetting robot for microtiter plates, a pump, and data processing. It could be demonstrated for a glutamate racemase from *Lactobacillus fermentii*, expressed in *Escherichia coli*, serving as model enzyme, that its activity can be determined from the time-dependent change of the optical rotation using L-glutamate as substrate. Thus, the specific activity was determined to 111.4 mdeg/min which corresponds to 45.7 μmol/min per mg purified enzyme. Moreover, a protocol was developed that allows the measurement of racemase activity from 96-well microtiter plates using purified enzymes. Thus, the method described can be used to determine racemase activity in an automatic manner. It should be also applicable for the screening of enzyme libraries created by directed evolution.

Racemases (EC 5.1.*x.y.*) provide organisms with the possibility to interconvert enantiomers, thus allowing them to use both enantiomers of a compound as nutrient or intermediate for catabolism.[1] Most α-amino acid racemases require a cofactor such as pyridoxal 5'-phosphate for catalytic activity. Glutamate racemase (EC 5.1.1.3) from *Lactobacillus fermentii* is one of the few enzymes that are cofactor-independent,[2] and it catalyzes the interconversion of the D- and L-enantiomers of glutamate with little or no activity toward other α-amino acids[3].

Beside their natural function, racemases are also important for biocatalysis.[4] In kinetic resolutions of racemates with stereoselective enzymes, the maximum theoretical yield of each enantiomer is 50%. To achieve higher yields, the nonwanted enantiomer must be racemized. In some cases, chemical racemization is possible, but an alternative is the use of a racemase allowing for a dynamic kinetic resolutions. These have been described for the synthesis of optically pure α-amino acids using, for example, combinations of hydantoinase, carbamoylase, and a racemase[5,6] and for the synthesis of optically pure mandelic acid.[7]

Modern molecular biology methods, such as cloning and expression of enzymes from "noncultivated" microorganims,[8−10] and the directed evolution[11,12] of proteins boost the number of available biocatalysts or mutants derived therefrom, and consequently, methods for their rapid and reliable characterization are required.

For α-amino acids and α-hydroxy acids, racemase activity can be determined by coupling their activity with an L-specific α-amino acid or alcohol dehydrogenase in connection with a dye.[13] Alternatively, rather expensive techniques based on circular dichroism have been described.[14] To reduce the costs for expensive equipment or the requirement of specific additional enzymes such as dehydrogenases, a generally and broadly applicable method would be desirable. One possibility is the determination of optical rotation values by polarimetric measurements. However, current equipment requires rather large amounts of substrates and enzymes and the measurement of numerous samples is very time-consuming and not automatized.[7]

In this paper, we describe a novel format for the direct determination of racemase activity based on polarimetric measurement in a microcuvette with automatized sampling from microtiter plates and data processing. This method can be performed in a medium-throughput format and thus allows the characterization of a broad number of biocatalysts.

EXPERIMENTAL SECTION

Reagents and Materials. L-Glutamate was purchased from Fluka (Buchs, Switzerland) as sodium salt.

For large-scale purification of the racemase, TALON-CellThru material (BD-Clontech, Palo Alto, CA) was used according to the

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10.1021/ac035012s CCC: $27.50 © xxxx American Chemical Society
Published on Web 00/00/0000

PAGE EST: 4.7

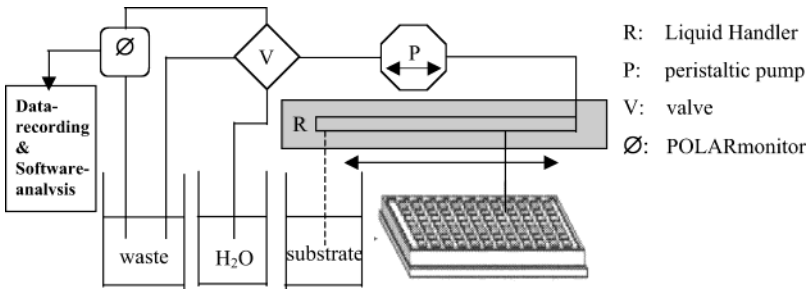

Figure 1. Setup of the device for the medium-throughput determination of racemase activity.

manufacturer's manual. The simultaneous purification of 96 enzyme samples was done with the TALON 96-well purification plate (BD-Clontech) for His-tagged proteins. For higher accuracy and time-saving, all pipetting steps were performed with a Tecan MiniPrep75 pipetting robot (Tecan, Crailsheim, Germany).

Optical rotation was determined using the POLARmonitor (IBZ-Messtechnik, Hannover, Germany), connected with a Liquid Handler 221 XL (Gilson) by a peristaltic pump (Minipuls 3, Gilson), Figure 1. The system was operated by the software X-Tray 3.0 (Gilson).

Cloning of the Glutamate Racemase into a Rhamnose-Inducible Expression Vector. The gene encoding glutamate racemase (Gene Bank accession code L02916) from *L. fermentii* cloned into the pUC18 vector was used as starting material. After isolation of plasmid DNA (QIA Quick Spin Miniprep Kit, Qiagen, Hilden, Germany) from *Escherichia coli* DH5α, the gene encoding the racemase was amplified by PCR with insertion of a C-terminal 6x His-Tag. The fragments were digested with restriction enzymes *Nde*I and *Bam*HI, purified (QIA Quick PCR Purification Kit, Qiagen) and ligated into a rhamnose-inducible expression vector.[15] Positive clones were confirmed by DNA sequencing using standard methods. Plasmids were then transformed into *E. coli* JM109 for racemase production.

Expression of the Glutamate Racemase. Cells were grown in *E. coli* JM109 in 2 mL in 96-deep well plates at 37 °C and 200 rpm shaking for 12 h. A total of 700 μL of LB medium was inoculated with 20 μL from a 200-μL overnight culture prepared in standard microtiter plates. These 720-μL minicultures were induced after 4-h cultivation time by adding 0.2% (w/v) L-rhamnose. After 12 h, cells were harvested by centrifugation (20 min, 1750*g*) and disrupted by three freeze/thaw cycles. Cell debris was removed by centrifugation (20 min, 1750*g*), and the supernatant containing racemase was used for further steps.

96-Well Purification. With the TALON 96-well purification plate, a simultaneous isolation of 96 different enzymes is possible. All following steps were performed at 4 °C. The standard protocol from BD-Clontech was modified as follows: after prequilibration of the binding material with 2 × 300 μL of binding buffer (50 mM phosphate buffer, 300 mM NaCl, pH 8.0), 300 μL of the supernatant was added to the plate, and binding of the protein was allowed for 15 min with intermediate shaking every 2−3 min. Nonbinding protein was removed by centrifugation (1 min, 700*g*), and the loading procedure was repeated with 300 μL of the supernatant. After the second loading step, the material was washed by adding 10 × 300 μL of the binding buffer followed by centrifugation (1 min, 700*g*) after each washing step. Elution of the purified racemase was performed by adding 300 μL of elution buffer (200 mM Tris-HCl, 150 mM imidazole, pH 8.5) followed by centrifugation (2 min, 700*g*) into a new 96-well plate.

SDS−PAGE and Protein Content Determination. Fifteen microliter aliquots of samples (supernatant, flow through, washing steps, and purified racemase solutions) were mixed with 5 μL of 4× SDS−sample buffer. After heating to 95 °C for 5 min, proteins were separated on polyacrylamide gels (12%) with a stacking gel (4%). Protein bands were visualized by silver staining at room temperature on a platform shaker. First, the gel was incubated for 1 h in the fixing solution (50% v/v ethanol, 10% v/v acetic acid, in distilled water) before equilibration for 30 min in the incubation solution (30% v/v ethanol, 830 mM sodium acetate, 13 mM sodium thiosulfate, 0.25% v/v glutaraldehyde, in distilled water) followed. After 3× washing for 10 min with distilled water, the gel was stained for 20 min in the silver solution (18 mM silver nitrate, 0.02% v/v formaldehyde, in distilled water) without shaking. Another 10-min washing step with distilled water followed, before the gel was incubated in a developing solution (250 mM sodium carbonate, 0.01% v/v formaldehyde, in distilled water) for 1−5 min. This was stopped by adding a 50 mM EDTA solution. Protein content was determined using the Bradford method.

Polarimetric Microtiter Plate Assay. The 96-well microtiter plate with 300 μL of the purified glutamate racemase samples was tape-sealed and transferred to the worktable of the Liquid Handler 221XL. Before each measurement, 50 μL of substrate was added to the sample by the Liquid Handler and mixed 3 times by pipetting. This solution was pumped into the measurement cuvette of the POLARmonitor using a peristaltic pump. The change in optical rotation was then monitored for 9 min. Next, the cuvette was washed for 2 min with distilled water before the next sample was measured.

Calculation of Racemase Activity and Specific Activity, Unit Definition. The monitored decrease in optical rotation *k* (Δα/min) was calculated from the slope of the initial rate (Figure 3B). From this, the specific activity of the racemase (in mdeg/min·mg of protein) was calculated using eq 1. The specific activity

$$\text{specific activity}\left[\frac{\text{mdeg}}{\text{min} \times \text{mg}}\right] = \frac{\text{charge in optical rotation } (\Delta\alpha)}{\text{enzyme concentration} \times \text{enzyme volume}}\left[\frac{\text{mdeg/min}}{\text{mg/mL} \times \text{mL}}\right] \quad (1)$$

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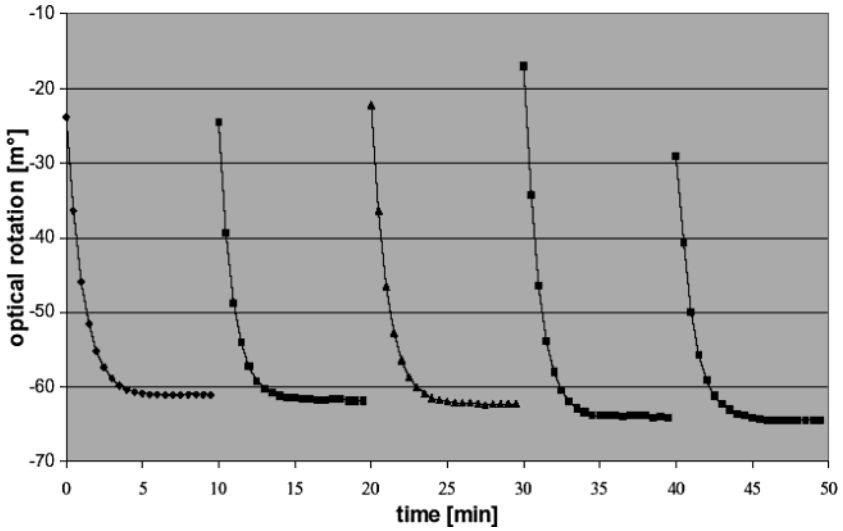

Figure 2. Accuracy and reproducibility of the system using L-glutamate (50 μmol) in the absence of racemase.

(in μmol/min·mg of protein) was calculated using eq 2. The

$$\text{specific activity}\left[\frac{\mu\text{mol}}{\text{min} \times \text{mg}}\right] = \frac{\text{charge in optical rotation } (\Delta\alpha) \times 0{,}41}{\text{enzyme concentration} \times \text{enzyme volume}} \left[\frac{\text{mdeg/min} \times \mu\text{mol}}{\text{mg/mL} \times \text{mL} \times \text{mdeg}}\right] \quad (2)$$

experimentally determined value of optical rotation at a L-glutamate concentration of 50 μmol was −61 mdeg (see Results and Discussion). From the initial rate determinations and a maximum consumption of 50% L-Glu, it was calculated that 1° corresponds to 0.41 μmol of L-Glu.

One unit (U) of racemase activity corresponds to the racemization of 1 μmol of L-Glu/min.

RESULTS AND DISCUSSION

Setup of the Device. To achieve a determination of optical rotation values, a setup for the handling of samples in a microtiter plate (MTP) format was developed (Figure 1). This is composed of the polarimeter device POLARmonitor, containing a microcuvette with 40-μL working volume, connected to a pipetting robot (Liquid Handler 221XL). Samples taken from the MTP are pumped to the polarimeter using a peristaltic pump. A four-way valve allows switching between measurement mode, discarding samples into a waste chamber, and washing of the cuvette with distilled water. Data collected by the polarimeter are recorded and processed using the software supplied by the manufacturer of the Liquid Handler. Thus, handling of 96 samples in a microtiter plate, addition of substrate to start the reaction, and monitoring of absolute optical rotation values as well as kinetic measurements are possible. In addition, the use of the POLARmonitor microcuvette with an accuracy in the millidegree scale allows the use of small sample volumes at low substrate concentrations.

Determination of Glutamate Racemase Activity. To verify the applicability, accuracy, and reproducibility of this system, racemization of L-glutamate using glutamate racemase from *L. fermentii* was chosen as model system, as this enzyme can be efficiently expressed recombinantly in *E. coli* and does not require any cofactor. In the first step, 10 samples with identical L-glutamate concentrations (142 mM, 50 μmol in total) were measured over 10 min (Figure 2 shows the first five runs). The results show an adjustment time of ∼3 min/sample required to achieve a stable value of optical rotation. The value for this L-glutamate concentration is ∼−61 mdeg. The reported rotation (α_{546}) for the sodium salt of L-glutamate given in the description of the supplier (Fluka, Switzerland) is −4.8° (c = 1.2 in H_2O, 25 °C). The difference between the measured and the literature values is in part due to a different concentration used here (c = 2.7 in H_2O) but is mostly related to the use of another measurement principle of the POLARmonitor (Faraday modulation). The slight decrease of the final rotation value (from −61 to −69 mdeg) can be possibly attributed to insufficient washing times between consecutive measurements leading to a low but measurable step-by-step increase in L-Glu concentrations. However, the determination of the absolute optical rotation value is not the major aim of this method, as for the determination of enzymatic activity, initial rates of the change in optical rotation values with time are the major target.

Initially, glutamate racemase obtained after expression in *E. coli* was directly used from cell lysate supernatants after removal of cell debris. Unfortunately, strong variations in optical rotation values were observed (data not shown), presumably due to the presence of media components and their degradation by impuring enzymes (e.g., proteases) resulting in the formation of optically active compounds. To avoid this, the glutamate racemase was purified using the TALON CellThru material, taking advantage of the 6× His-tag cloned C-terminal to the mature racemase. Now, the time course of racemization of optically pure L-Glu (100 μmol) with purified glutamate racemase (18.5 μg) could be monitored. In the control reaction, no change in optical rotation was found in the absence of either substrate or enzyme (Figure 3A). Also, imidazol used for protein elution did not affect the measurement of optical rotation and enzyme activity (data not shown). After

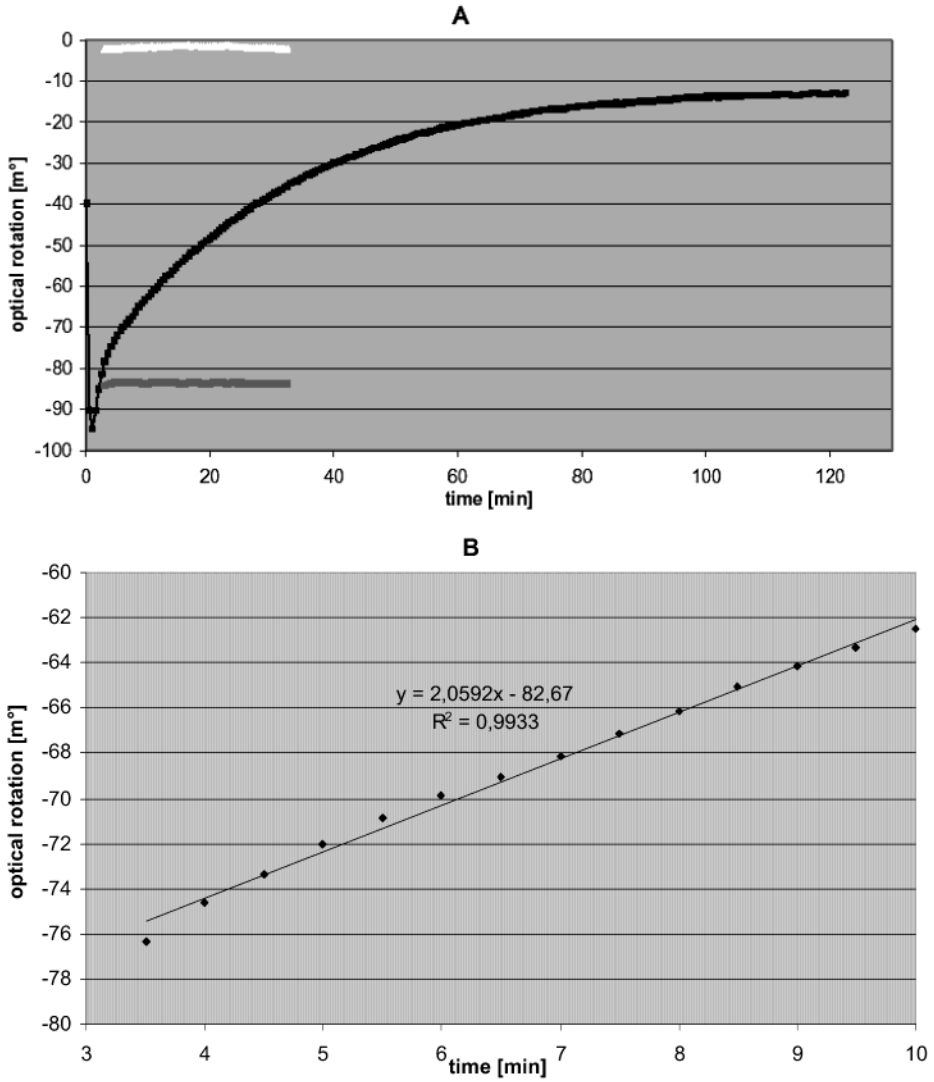

Figure 3. (A) Time course of L-glutamate racemization using purified enzyme. (■), 18.5 μg of purified L-Glu racemase, 100 μmol of L-glutamate, 200 mM Tris-HCl, pH 8.5; (gray solid box), control without enzyme; (□) control without glutamate. (B) Linear range (first 10 min) used for the calculation of specific enzyme activity.

2-h reaction time, no further decrease in optical rotation was found; however, the value of optical rotation did not reach zero (Figure 3A). This can be explained by minor contributions of the protein solution to the rotation value.

Kinetic analysis of the first 10-min reaction time reveals an excellent linear relationship (Figure 3B, $R^2 = 0.9933$). From the slope of the initial rate, the decrease in optical rotation k ($\Delta\alpha$/min) was calculated to 2.06 mdeg/min. From eq 1, the specific activity of the racemase was calculated to 111.4 mdeg/min·mg of racemase. Using eq 2, a specific activity of 45.7 U/mg enzyme was determined. These values are in the same micromole range as reported in the literature for purified glutamate racemases (7.7[3] and 158[13] U/mg). We assume, that the discrepancy between these two literature values are due to different degrees of purity of the glutamate racemases used.

In the next step, five different enzyme concentrations (0.1−20 μg of protein) were used at fixed substrate concentration (100 μmol) and a good correlation was found (data not shown). Variation of substrate concentration at a given enzyme concentration suggested substrate inhibition at high L-Glu concentration above 100 μmol (Table 1).

Table 1. Glutamate Racemase Activity Determined at Increasing L-Glutamate Concentrations Using 10 μg of Purified Enzyme

L-glutamate (μmol)	slope k (mdeg/min)	spec activ (mdeg/min·mg)	spec activ (μmol/min·mg)
10	1.02	102	41.8
30	1.06	106	43.5
70	1.14	114	46.7
100	1.19	119	48.8
150	0.86	86	35.3
200	0.61	61	25.0

Finally, the applicability of the method for medium-throughput determination of racemase activity was investigated. A microtiter plate containing 96 samples of this glutamate racemase were analyzed. All samples were cultivated in a deep-well plate followed by parallel purification of the 96 samples in a MTP using the modified manufacturer's protocol (see Experimental Section), which includes cell culture, cell disruption, protein purification, and activity determination. The efficiency of the purification step was also confirmed by SDS−polyacrylamide gel electrophoresis

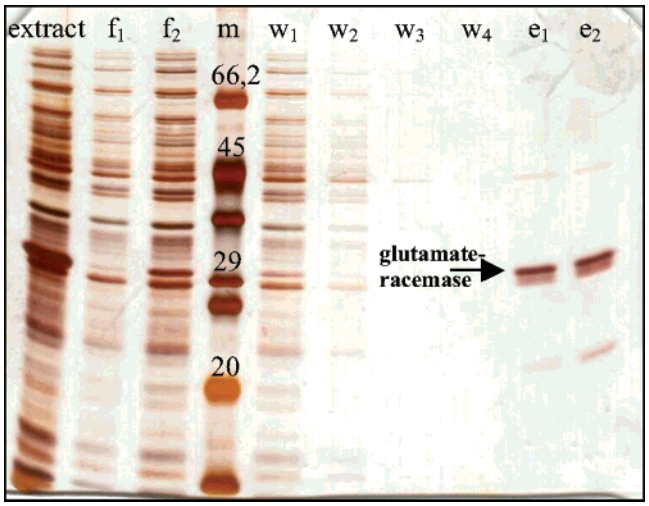

Figure 4. Silver-stained SDS−PAGE to verify purification of the overexpressed 29-kDa glutamate racemase in the 96-well scale: (f) flow through; (m) marker in kDa; (w) washing step; (e) elution step.

Scheme 1. Protocol for Automatized Measurement of Optical Rotation Values in a 96-Well Format

- *Start*
- 1 s prequilibration of the syringe
- add substrate (50 µl)
- 10 s wait
- mix (50 µl) (2 x)
- 10 s wait
- pump sample (350 µl) into the cuvette
- equilibration of the syringe
- 9 min collection of data, each 30 s
- 2 min wash the cuvette (95x)
- *End*

(Figure 4) and protein content measurement (3.7 μg/well). Using the pipetting program for the sequential determination of racemase activity (Scheme 1), all 96 samples were subjected to activity determination. From the data collected, an average change in optical rotation of 0.37 mdeg/min (±0.13) was calculated, which corresponds to a specific activity of 40.7 U/mg of protein (±14.3). This closely matches the value given above for a one-step measurement not performed in a microtiter plate format.

CONCLUSIONS

In this paper, a versatile method for the determination of α-amino acid racemase activity, as exemplified for a recombinant glutamate racemase, is described. The overall setup with connection of the microcuvette polarimeter to a microtiter plate, pipetting robot, and data processing enables the automatic screening of 96 samples. The time required to measure each sample was ∼11 min, which is equivalent to 17 h, to analyze the entire plate. However, according to Figure 3B, shorter analysis times should be also sufficient and we estimate that 3 × 96 samples could be measured per day.

The major advantage of this system is its automization as the conventional determination of optical rotation values is performed manually. Furthermore, due to the small cuvette volume of 40 μL (overall sample volume required, 350 μL), smaller amounts of substrate (≥10 μmol) or enzyme (∼2−3 μg) are needed. The fact that purification of the enzyme was necessary here is mostly due to the use of a recombinant enzyme expressed intracellularly in *E. coli*. This might not be necessary for enzymes produced by secreting organisms or commercial enzyme powders.

The method is not restricted to activity determination and substrate profiling of racemases. In principle, the same format can also be used for the quantitative determination of chemical racemization methods, especially when many different reaction conditions have to be investigated. Also, the screening of enzymes (such as alcohol or amino acid dehydrogenases, lipases, esterase, epoxide hydrolases, etc.) converting prostereogenic or meso (and therefore optically inactive) compounds into chiral compounds can be investigated using the same setup and protocols.

ACKNOWLEDGMENT

We thank Prof. Rolf Schmid and Sandra Barth (Stuttgart University, Stuttgart, Germany) for providing the glutamate racemase gene and the Lonza AG (Visp, Switzerland) and Degussa AG (Project House Catalysis, Frankfurt, Germany) for financial support.

Received for review August 28, 2003. Accepted November 8, 2003.

AC035012S