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Determination of biocatalyst consumption in an aminopeptidase process using automated sample preparation and high-performance liquid chromatography

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ABSTRACT

A rapid and sensitive method has been developed for the determination of the biocatalyst consumption in the chemo-enzymic production of optically pure natural and synthetic α-H-amino acids. It is based on automated sample preparation from an enzymic reaction mixture, reversed-phase high-performance liquid chromatographic separation, post-column reaction and fluorimetric detection. The assay procedure has been applied to the enzymic conversion of racemic norvaline amide into L-norvaline, catalysed by an L-specific aminopeptidase from *Pseudomonas putida*. Both norvaline amide and norvaline can be analysed in a single assay in the low nanogram range. The method yields reproducible results and requires 30 min from the time of sampling the enzymic reaction mixture to quantitation. The reaction mixture is automatically sampled and analysed several times during the course of the reaction. With the results obtained a conversion curve can be constructed from which the exact biocatalyst consumption can be calculated. By adaptation of the mobile phase, the method can also be applied to other amino acid amides used as substrates in the aminopeptidase reaction.

INTRODUCTION

Optically pure amino acids are becoming increasingly important as intermediates for the production of pharmaceuticals, food additives and agrochemicals. Production methods include fermentation, asymmetric synthesis and resolution processes. Enzymic resolution methods in particular are powerful tools for the production of optically pure D- and L-amino acids. One of the routes to these compounds is through organic synthesis of racemic α-amino acid amides, followed by the use of a broad-specificity peptidase to achieve resolution on a large scale [1,2]. To obtain optically pure amino acids, 100% conversion of the L-amino acid amide into the L-amino acid is necessary.

As enzymes are very sensitive to the quality of the substrate, the consumption of the biocatalyst consumption will vary according to the amount of the impurities present in the substrate (salts, ammonia or organic solvents). Because of this variation, it is desirable to determine the biocatalyst consumption for each sub-

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strate batch. An L-specific aminopeptidase from *Pseudomonas putida* was used as biocatalyst for the conversion of racemic norvaline amide (Nval-NH_2) into L-norvaline (L-Nval). The enzyme productivity can be determined by quantitating the substrate and the product of the reaction. High-performance liquid chromatography (HPLC) is a suitable technique for this type of assay [3].

This paper describes an analytical method for the measurement of aminopeptidase productivity. The method consists of an automated sampling device for a laboratory-scale bioreactor. This device carries out the following steps: sampling, dilution, mixing and filtration. The filtrate is then injected into an HPLC system, which is coupled on-line with the sampling device. By means of reversed-phase chromatography, the substrate and product are separated and, after post-column reaction, selectively detected by means of fluorimetry. The results obtained supply the production plant with the necessary information concerning the biocatalyst consumption.

EXPERIMENTAL

Materials

Nval was purchased from Sigma (St. Louis, MO, U.S.A.), and Nval-NH_2 was synthesized in our laboratory [1]. The pairing ion, laurylsulphonate, *o*-phthalaldehyde (OPA), HPLC-grade 2-propanol and ethanol were obtained from Merck (Darmstadt, Germany). 2-Mercaptoethanol was supplied by Fluka (Buchs, Switzerland). Water was purified by a Milli-Q system. All other chemicals were of analytical-reagent grade.

The enzyme sludge was obtained by fermentation of *P. putida* ATCC 12633 in complex media, concentration by different centrifugation steps, and homogenization of the whole-cell biocatalyst, *e.g.* by the addition of toluene.

Instrumentation

A Gilson (Villiers-le-Bel, France) Model 232-401 automatic sample processor was used for sample preparation. Two rack positions were used for placing two thermostatted 100-ml reaction barrels on a magnetic stirrer. One rack was used for making dilutions of the reaction mixture and one rack for filtration of the diluted samples. For filtration, a cartridge was used filled with a wad of cotton wool. After filtration, the tray containing the cartridge was shifted to the other side of the rack, whereupon the filtrate was injected into the HPLC column.

A Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 1081B pump was used for solvent delivery, and injection was performed with a Rheodyne (Cotati, CA, U.S.A.) 7010 injection valve. The injection loop had a 20-μl capacity. The column used was a Nucleosil 120-C_{18} (250 mm × 4.0 mm I.D., 5 μm). The flow-rate was 1.0 ml/min, and the column temperature was kept at 40°C.

For reaction detection, the OPA reagent was added to the column effluent via a mixing tee-piece. Post-column addition of reagent was effected with a Gilson

Model 302 pump at a flow-rate of 1.0 ml/min. The OPA reaction was carried out in a coiled capillary stainless-steel tube (12 m × 0.35 mm I.D., coil diameter 12 mm) at 40°C. The fluorophores were monitored with a Hitachi (Tokyo, Japan) Model F1000 fluorescence detector using an excitation wavelength of 340 nm and an emission wavelength of 380 nm. Quantitation was performed with a Hewlett-Packard 3350 laboratory automation system.

Eluent, reagent and enzymic reaction mixture

The eluent was a mixture of sodium phosphate buffer (0.01 *M* Na^+, pH 3.0), sodium laurylsulphonate (0.5 m*M*) and 2-propanol (15%). The OPA reagent consisted of potassium borate buffer (0.4 *M*, pH 10.0), OPA (6 m*M*), 2-mercaptoethanol (MCE) (0.1%) and ethanol (1%). For the preparation of the enzymic reaction mixture, the racemic Nval-NH_2 solution was heated to 40°C, and different amounts of enzyme were stirred into the substrate solutions. At different time intervals, representative aliquots were then taken from the reaction mixture by means of the automated sampling device. The aliquots were diluted with 0.1 *M* phosphoric acid to stop the enzymic reaction.

RESULTS AND DISCUSSION

Automatic sample preparation

For the automatic analysis of the concentration of Nval-NH_2 and Nval in the reaction mixture as function of the reaction time, the following problems had to be solved: (i) taking a representative aliquot from the reaction mixture; (ii) lowering the concentrations of the substrate and the reaction product in the reaction mixture to a level suitable for HPLC analysis; and (iii) making the solution to be injected particulate-free.

After addition of the biocatalyst to the substrate solution, the solution flocculated. To take an aliquot from this solution, we equipped the sampling device with the largest bore needle available from the manufacturer, *i.e.* 0.5 mm. For the selective measurement of Nval and Nval-NH_2 in the reaction mixture, we chose fluorimetric detection after post-column labelling of the primary amines. The choice of this sensitive detection technique, however, implied that a dilution of the aliquot from the reaction mixture by a factor 500 had to be made. Taking into account the maximum volume of the tubes (7 ml), we decided to make the dilution in two steps, by factors of 20 and 25. This was accomplished by taking an aliquot of 350 μl from the reaction mixture. We studied several possible methods of diluting the samples. An overview of the different dilution modes, together with the associated coefficients of variation (C.V.) obtained for the HPLC assay, is given in Table I. It can be seen that, starting from injection without sample pretreatment, the C.V. increases as the number of preparation steps increases (mixing and mixing plus filtration). Initially, the solutions were mixed by aspirating and dispensing the solution in the vial. Repetition of this action resulted in a

TABLE I

REPRODUCIBILITY OF THE HPLC SYSTEM, INCLUDING THE AUTOMATED SAMPLE PREPARATION

The reproducibility was tested by measuring the peak area of Nval. The concentration was 0.9 mg/50 ml.

Preparation	Coefficient of variation (%)
Injection (without sample preparation)	0.4 (n = 10)
Dilution (one mix[a])/injection	3.9 (n = 9)
Dilution (three mixes[a])/injection	2.4 (n = 10)
Dilution (three mixes[a])/filtration/injection	3.9 (n = 10)
Dilution (three mixes[b])/filtration/injection	4.2 (n = 8)

[a] Mixing was performed by aspirating and dispensing the diluted solution.
[b] Mixing was performed by aeration of the diluted solution.

lower C.V. To visualize the homogeneity of the diluted solution, we added phenolphthalein to the solution.

Even after mixing three times, the solution did not show an even colour distribution. Aeration of the solution via the needle resulted in a more homogeneous solution (as indicated by the colour), when compared with the case of an aspirating/dispensing action. The C.V. obtained for the two mixing modes were comparable. Mixing by means of aeration was finally selected as the mixing mode for the sample preparation. The particulate matter present in the final diluted solution was removed by passing the solution through a cartridge filled with a wad of cotton wool. Thereafter the filtrate was injected into the HPLC system.

Choice of the HPLC method

For the measurement of Nval and Nval-NH_2, a chemical derivatization step was included in the analysis. The main reason for this was to increase the specificity of the analysis, because initial experiments indicated that application of UV detection at low-wavelength settings (200–220 nm) resulted in a lot of interfering peaks in the chromatogram. Chemical derivatization can be applied in the pre- or post-column mode. Because of the varying composition of the substrate batches, especially the concentration level of the impurities, we preferred post-column derivatization. In an earlier paper [4], we described an ion-pair reversed-phase HPLC method for the analysis of some aliphatic amino acids, together with the corresponding amino acid amides. This method gives now optimal separations for Nval, Nval-NH_2 and potentially interfering primary amines, by varying the concentration of the ion-pairing reagent (laurylsulphonate) and the content of organic solvent (2-propanol) in the mobile phase.

For the post-column labelling of Nval and Nval-NH_2, the OPA reagent was chosen. A representative chromatogram of a sample from the enzymic reaction mixture is given in Fig. 1. The molar fluorescence intensities (relative units) ob-

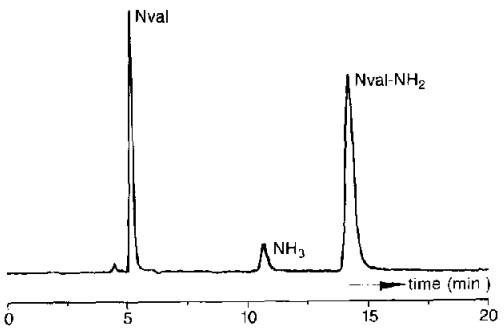

Fig. 1. Chromatogram of a sample from the biocatalytic conversion of Nval-NH_2.

tained for the OPA derivatives of Nval and Nval-NH_2 were 100 and 9, respectively. Quantitative determinations were carried out by comparing the peak areas of samples with those of standard solutions, employing the external standard method. The linearity of the response was established over the range 6–27 nmol for Nval-NH_2 and 0.6–7 nmol for Nval. Linear regression analysis from standard curves indicated that the correlation coefficients for both compounds were higher than 0.9994.

Determination of the biocatalyst consumption

In order to determine the minimum amount of enzyme required for the enzymic resolution process, the course of the reaction, starting with racemic Nval-NH_2 in combination with different enzyme dosages, was followed using the described automated sampling system. As an example, several conversion curves related to different enzyme amounts are shown in Fig. 2. The minimum amount needed for complete conversion, within a certain period of time, of the L-enantiomer in the racemic amide mixture was then chosen from these curves and applied to the biocatalytic conversion process in the production plant.

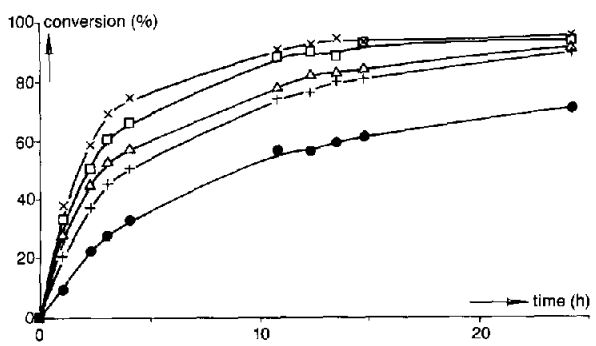

Fig. 2. Conversion of racemic Nval-NH_2 as a function of reaction time at several enzyme concentrations: (●) E_1; (+) $2E_1$; (△) $3E_1$; (□) $4E_1$; (×) $5E_1$.

REFERENCES

1 E. M. Meijer, W. H. J. Boesten, H. E. Schoemaker and J. A. M. van Balken, in J. Tramper, H. C. van der Plas and P. Linko (Editors), *Biocatalysis in Organic Synthesis*, Elsevier, Amsterdam, 1985, p. 135.
2 W. H. Kruizinga, J. Bolster, R. M. Kellogg, J. Kamphuis, W. H. J. Boesten, E. M. Meijer and H. E. Schoemaker, *J. Org. Chem.*, 53 (1988) 1826.
3 B. N. Jones, P. P. Tamburini, A. P. Consalvo, S. D. Young, S. L. Lovato, J. P. Gilligan, A. Y. Jeng and L. P. Wennogle, *Anal. Biochem.*, 168 (1988) 272.
4 A. Duchateau and M. Crombach, *Chromatographia*, 24 (1987) 339.