Carlsberg Res. Commun. Vol. 49, p. 585–590, 1984

DETERMINATION OF D-AMINO ACIDS. I. HYDROLYSIS OF DNP-L-AMINO ACID METHYL ESTERS WITH CARBOXYPEPTIDASE-Y

by

PETER MARFEY[1] and MARTIN OTTESEN

Department of Chemistry, Carlsberg Laboratory,
Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby

[1] Permanent address: Department of Biological Sciences,
State University of New York at Albany, Albany, NY 12222, USA

Keywords: D-amino acids, racemization, DNP-derivatives, carboxypeptidase-Y

L- and D-amino acids were treated with fluorodinitrobenzene to form DNP-derivatives. The latter were esterified with methanol in the presence of dry HCl gas to the corresponding methyl esters. Treatment of the ester mixture with carboxypeptidase-Y resulted in the hydrolysis of only L-esters. All D-esters and some L-esters were unaffected. The produced DNP-L-amino acids could be separated from DNP-D-amino acid methyl esters by high performance liquid chromatography allowing quantitation of the D-isomers. When a test mixture consisting of Ala, Asp, Glu, Met and Phe, present in different proportions as L- and D-isomers, was subjected to the above procedure, all D-amino acids could be quantitated. The method allows detection of D-amino acids in the nanomole range.

1. INTRODUCTION

There is a growing interest in the quantitative determination of D-amino acid residues in proteins and peptides because of the recent findings that many proteins and peptides can undergo partial racemization as a result af aging, irradiation with ionizing radiation, thermal treatment or treatment with certain chemicals that promote racemization (1, 4).

In the present paper a simple way of separating and quantitating some L- and D-amino acids is described. The basis of the method is the use of an enzyme such as carboxypeptidase Y (3, 8) which hydrolyses esters of only L-isomers and not D-isomers. Thus, when a mixture of L- and D-DNP-amino acid methyl esters is treated with the enzyme, D-esters are unaffected. The hydrolysis mixture is then subjected to HPLC resulting in a separation of D-esters from the corresponding L-acids. The DNP-chromophore is sufficiently stable and strongly absorbing to permit separation and quantitation of D-amino acids in the nanomolar range.

2. MATERIALS AND METHODS

2.1. Materials

CPD-Y was obtained from Carlsberg Biotechnology Ltd. Subtilisin A was from Novo Industri Ltd. Pronase was from Calbiochem., USA. L-

Abbreviations: CPD-Y = carboxypeptidase-Y; DMF = N,N-dimethylformamide; DMSO = dimethylsulfoxide; DNP = 2,4-dinitrophenyl; FDNB = 1-fluoro-2,4-dinitrobenzene; HEPES = N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid; HPLC = high performance liquid chromatography; TEAP = triethylammonium phosphate; TLC = thin-layer chromatography. Other abbreviations are according to the guidelines of the IUPAC-IUB, Commision of Biochemical Nomenclature.

Springer-Verlag 0105-1938/84/0049/0585/$01.20

and D-amino acids, DNP-L-amino acids and 1-fluoro-2,4-dinitrobenzene were purchased from Sigma, USA. Some of the DNP-amino acids and all DNP-amino acid methyl esters were synthesized as described below. Other chemicals and solvents were of reagent grade.

2.2. Methods

2.2.1. Synthesis of DNP-amino acids (5)

The following compounds were synthesized: DNP-L-Ala, DNP-D-Ala, DNP-L-Arg, DNP-D-Asp, DNP-L-Asp, DNP-D-Glu, DNP-L-Glu, DNP-D-Met, DNP-L-Met, DNP-D-Phe, DNP-L-Phe, DNP-D,L-Ser, DNP-D,L-Thr and DNP-derivatives of D,L mixtures of Asp, Glu and Phe. All were synthesized essentially in the same manner as described for DNP-L-Phe: L-Phe (200 mg, 1.2 millimoles) was placed in an Erlenmeyer flask, solid FDNB (225 mg, 1.2 millimoles) was added followed by 20 ml of acetone. Solid $NaHCO_3$ (202 mg, 2.4 millimoles) was added followed by a slow addition (10 min) of 10 ml water. Contents were stirred for 0.5 hour at 30-40°C. Another identical portion of solid $NaHCO_3$ was added and the contents stirred at the same temperature for another hour. After concentration to 10-15 ml, a precipitate was formed which was filtered, carefully washed with water and dried in the air and in the dark. The yields of the air-dried products ranged from 73% (DNP-D,L-Asp) to 99% (DNP-D,L-Thr).

2.2.2. Synthesis of DNP-amino acid methyl esters (2)

Samples (15-20 mg) of DNP-amino acids were placed into plastic cups. About 1.5 ml methanol was added resulting in nearly complete solubilization of the samples. Dry HCl gas was bubbled through the solutions for 5 min. Cups were closed and kept for about 3 hours. Thereupon, the contents were evaporated to dryness first in a stream of air and then in a vacuum desiccator over solid NaOH pellets. The conversion to methyl esters was quantitative as judged by chromatography (see section *2.2.3*). Both the acids and the corresponding methyl esters have a strong absorption maximum at 350 nm with a $\varepsilon_M \cong 1.5 \times 10^4$.

2.2.3. Chromatography

TLC was done with pre-coated plastic sheets containing 0.25 mm layers of silica gel impregnated with fluorescent indicator UV 254 (Polygram Sil G/UV 254). Ethyl acetate was used as a developing solvent. HPLC was done with Waters Associates equipment employing a C-18 column (8 mm × 10 cm) with a linear gradient of solution A (50 mM-TEAP buffer, pH 3.0) and solution B (25 mM-TEAP buffer, pH 3.0, and 50% acetonitrile) from 50% B to 100% B in 5 minutes, and a flow rate of 2 ml/min. Alternatively, a linear gradient 25% to 50% acetonitrile in 50 mM-TEAP buffer (pH 3.0) was used during 40 min. Reading was done at 350 nm. Samples of DNP-amino acids or DNP-amino acid methyl esters were dissolved in DMSO, approx. 2 mg per ml, and 3-5 µl of these solutions were injected for HPLC.

2.2.4. Enzymatic ester hydrolysis

Hydrolysis of DNP-amino acid ester to the corresponding acid is accompanied by an increase in absorption, maximally at 370 nm. $\Delta\varepsilon_{370}$ was 2.46×10^3. This value was used for the calculation of the hydrolysis rates using the three enzymes, CPD-Y, subtilisin A (7) and pronase at 25 °C.

2.2.5. Determination of D-amino acids in a mixture of five amino acids

An artificial amino acid mixture (a mixture of 0.1 ml samples of 50 mM solutions in water of L- + D-Ala, L- + D-Asp, L- + D-Glu, L- + D-Met and L- + D-Phe, total mixture 0.5 ml containing 25 micromoles of amino acids) containing known amounts of D-amino acids was evaporated to dryness using 37 °C water bath and air stream. The residues were treated with 0.25 ml 1 M-$NaHCO_3$ (0.25 millimoles) followed by 0.5 ml of 1% FDNB in acetone (27 micromoles, ratio of FDNB to amino acid 1.1:1). The contents were mixed and heated at 50 °C for 1 hour. After cooling to room temperature, 0.2 ml of 2 M-HCl was added and the contents were dried over NaOH pellets in a vacuum desiccator. To the dry residues 1 ml of methanol was added, dry HCl gas was bubbled for 5 min, the vials were closed

and kept for 3 hours. The contents were evaporated and dried over NaOH pellets in a desiccator. The dry residues were dissolved in 0.4 ml DMSO.

50 µl samples of DMSO solution (3.13 micromoles) were added to 0.2 ml 50 mM-HEPES, pH 7.5, buffer and treated with 50 µl of CPD-Y solution (10.6 mg/ml in H_2O). The contents were mixed, kept at room temperature for 3 hours and then diluted with 0.3 ml DMSO yielding a solution containing 5.21 nanomoles of DNP derivatives per µl. Aliquots of 25 µl (130.3 nanomoles) was used for HPLC as described in section *2.2.3* using the 40 min. linear gradient. Experimental blanks containing all the components except the enzyme were also prepared and analyzed by HPLC.

3. RESULTS

3.1. Chromatographic characteristics of DNP-amino acids and the corresponding methyl esters

DNP-amino acids and the corresponding methyl esters are sufficiently stable compounds so that they can be handled under a variety of conditions except high pH (above 12) when they undergo slow racemization. They also contain a strongly absorbing yellow-orange chromophore which allows easy separation and quantitation by TLC or by HPLC. The R_f values for esters range from 0.8 to 0.9 and for acids from 0.1 to 0.2 as determined by TLC with ethyl acetate as the solvent.

In Table I are given HPLC elution times of DNP-amino acids and of the corresponding methyl esters. The elution times for the acids are consistently shorter than for the corresponding methyl esters. The most hydrophilic compound, both in the acid and in the ester series, is DNP-L-Arg and the most hydrophobic compound is di-DNP-L-Lys (in the acid series) and di-DNP-L-Tyr (in the ester series). All other compounds are eluted between these two extremes.

Table I.
HPLC elution times of DNP-amino acids and the corresponding methyl esters[a)]

Substance	Elution time (minutes)	
	Acid	Ester
DNP-L-Ala	6.62	9.77
DNP-D-Ala	6.62	9.77
DNP-L-Arg	3.91	6.13
DNP-D,L-Asp	6.26	9.13
di-DNP-L-Cys	8.00	18.5
DNP-D,L-Glu	5.02	9.81
DNP-Gly	5.56	8.32
DNP-L-His	7.83	10.80
DNP-L-Ile	9.43	15.77
DNP-L-Leu	9.49	15.78
di-DNP-L-Lys	10.83	19.03
DNP-L-Met	7.86	11.85
DNP-D,L-Met(SO)	4.33	7.00
DNP-D,L-Phe	9.08	14.33
DNP-L-Pro	6.77	9.71
DNP-D,L-Ser	4.17	6.70
DNP-D,L-Thr	5.33	7.73
DNP-L-Trp	8.68	12.50
di-DNP-L-Tyr	9.58	22.66
DNP-L-Val	8.36	12.94

[a)]Determined with C-18, 8 mm × 10 cm radial compression column (Waters Associates) using a linear gradient of solution A (50 mM-triethylammonium phosphate buffer, pH 3.0) and solution B (25 mM-triethylammonium phosphate, pH 3.0, and 50% acetonitrile) from 50% B to 100% B, 5 minutes, 2 ml/min, reading at 350 nm.

3.2. Enzyme hydrolysis of DNP-L-amino acid methyl esters

The specific activities of CPD-Y, subtilisin A and pronase are recorded in Table II. It is seen that under the conditions used each enzyme hydrolyzed only a limited number of DNP-L-amino acid methyl esters. CPD-Y hydrolyzed seven of them with specific activities ranging from 500 (DNP-D,L-Phe-OMe) to 2.0 (DNP-D,L-Met(sulfoxide)-OMe). This enzyme hydrolyzed only esters of the DNP-L-amino acids. Subtilisin A hydrolyzed 12, with the highest activity for DNP-L-Trp-OMe and the lowest for DNP-Gly-OMe. However, when two isomers of Ala were tested, it was found that the enzyme hydrolyzed both L- and D-esters, L-isomer 3 times faster than D-isomer. Pronase had low activity with DNP-L-amino acid methyl esters and, like subtilisin A, it hydrolyzed both isomers of Ala.

Table II.
Enzyme hydrolysis of DNP-amino acid methyl esters[a]

Substrate	Specific activity (μmoles substrate/mg enzyme/min)		
	CPD-Y	Subtilisin A	Pronase
DNP-L-Ala-OMe	9	14	1
DNP-D-Ala-OMe	0	5	2
DNP-L-Arg-OMe	0	0	0
DNP-D,L-Asp(-OMe)$_2$	9	17	1
di-DNP-L-Cys-OMe[b]	0	0	0
DNP-D,L-Glu(-OMe)$_2$	20	8	0.5
DNP-Gly-OMe	0	2	1
DNP-L-His-OMe	0	3	0
DNP-L-Ile-OMe	0	0	0
DNP-L-Leu-OME	0	0	0
di-DNP-L-Lys-OMe[b]	0	0	0
DNP-L-Met-OMe	hydrolyzed[c]	0	0
DNP-D,L-Met(SO)-OMe	2	20	0
DNP-D,L-Phe-OMe	500	9	0
DNP-L-Pro-OMe	0	36	0
DNP-D,L-Ser-OMe	0	14	0.4
DNP-D,L-Thr-OMe	0	0	0
DNP-L-Try-OMe	31	50	2
di-DNP-L-Tyr-OMe[b]	0	0	0
DNP-L-Val-OMe	0	5	0

[a]Specific activities were determined at 25 °C spectrophotometrically at 370 nm. At this wavelength ΔA per μmole ester hydrolyzed is + 2.46 × 10^{-3}. The assay mixture contained 0.8 ml 50 mM-HEPES, pH 7.5, buffer, 0.2 ml DMSO, 10 μl of ester solution (1-2 mg/ml DMSO) and 10-50 μl enzyme solution (5 mg CPD-Y/ml H_2O, 5 mg subtilisin A/ml H_2O, 10-20 mg pronase/ml H_2O made 50 mM in $CaCl_2$.
[b]These substrates precipitated during the assay
[c]Specific activity was not determined. However, it was hydrolyzed in the artificial five amino acid mixture (see below).

3.3. CPD-Y hydrolysis of a mixture of DNP-D- and DNP-L-amino acid esters

Mixtures consisting of the five amino acids, Ala, Asp, Glu, Met and Phe were derivatized with FDNB, esterified with MeOH and hydrolyzed with CPD-Y as described in section *2.2.5*. Each amino acid, however, was a known mixture of L- and D-isomers. The proportion of the D-isomer in each of the five samples was 0%, 5%, 10%, 20% and 100%.

A typical HPLC chromatogram of mixture 1 containing 80% L- and 20% D-isomer of each of the five amino acid derivatives is shown in Figure 1. All the five D-esters are separated. Esters of the L-amino acid derivatives are hydrolyzed to the corresponding acids which are well separated from the esters. CPD-Y does not seem to hydrolyze further β and γ methyl esters of Asp and Glu because the peaks corresponding to the free acids are nearly absent (DNP-L-Asp at 3.62 min and DNP-L-Glu at 4.53 min). The identity of all the peaks was established in separate HPLC runs.

Other chromatograms corresponding to 10% D- and 90% L-isomers and 5% D- and 95% L-isomers, respectively, are not shown, but they are very similar to the pattern of peaks shown in Figure 1, except for the size of peaks. The HPLC area corresponding to the D-esters in these chromatograms (Figure 2) are proportional to the amounts expected in the three mixtures which contained 5% (1.3 nanomoles), 10% (2.6 nanomoles) and 20% (5.2 nanomoles) of D-isomers. The slopes of the curves are slightly different because the ε_M values of the five esters are slightly different from the ε_M values of the corresponding

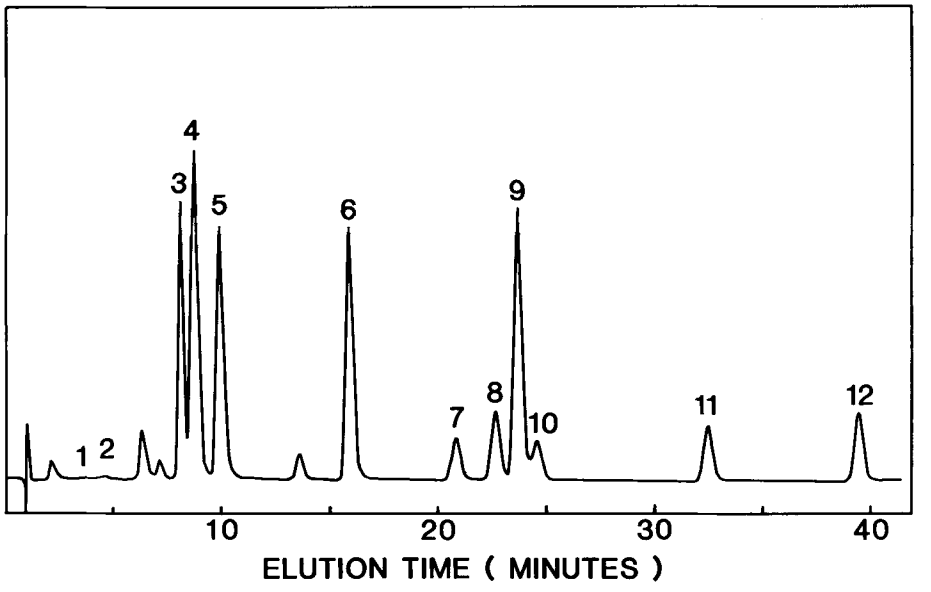

Figure 1. HPLC of a mixture containing Ala, Asp, Glu, Met and Phe, each amino acid being a mixture of 80% L- and 20% D-isomer. Peak 1, DNP-L-Asp, 3.62 min; peak 2, DNP-L-Glu, 4.53 min; peak 3, DNP-L-Asp-β-OMe, 8.10 min; peak 4, DNP-L-Ala, 8.76 min; peak 5, DNP-L-Glu-γ-OMe, 9.93; peak 6, DNP-L-Met, 15.93 min; peak 7, DNP-D-Asp $(-OMe)_2$, 20.91 min; peak 8, DNP-D-Ala-OMe 22.75 min; peak 9, DNP-L-Phe, 23.78 min; peak 10, DNP-D-Glu $(-OMe)_2$, 24.68 min; peak 11, DNP-D-Met-OMe, 32.58 min; peak 12, DNP-D-Phe-OMe, 39.59 min. Other peaks seen in the chromotogram are due to excess hydrolyzed reagent. Identification of the peaks was done by using known samples in separate HPLC runs. A linear gradient (25% to 50%) of acetonitrile in 50 mM-TEAP, pH 3.0, buffer during 40 min was used for HPLC (see section *2.2.5* for other details).

free acids at 350 nm, the wave length at which peak detection was done.

4. DISCUSSION

CPD-Y shows a remarkable selectivity for L-isomers of DNP-amino acid esters which it hydrolyses to the corresponding free acids. Because of this property, we have utilized it for quantitative estimation of either L- or D-isomers in various mixtures of the two.

The first step is the reaction with FDNB (5). It was first shown with L-Ala and D-Ala that no racemization occurs during the conditions of

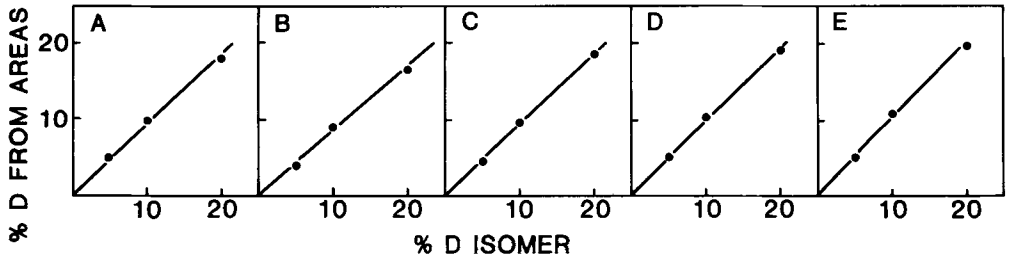

Figure 2. Relationship between % D-isomer determined from HPLC area and % D-isomer present in mixture 1 (80% L and 20% D), mixture 2 (90% L and 10% D) and mixture 3 (95% L and 5% D). Percent of D from areas was calculated as follows: % D = [D-area/(D-area + L-area)]×100. A, DNP-D-Asp $(-OMe)_2$ (slope 0.93); B, DNP-D-Glu $(-OMe)_2$ (slope 0.85); C, DNP-D-Ala-OMe (slope 0.92); D, DNP-D-Met-OMe (slope 0.95); E, DNP-D-Phe-OMe (slope 1.00). HPLC conditions are the same as in Figure 1.

this reaction. The results of the analysis of the five amino acid mixture support this conclusion. The next step is esterification with methanol (2). This can be done directly with the reaction mixture of the preceding step without first isolating DNP-amino acids. Under the conditions employed, esterification is quantitative (as proved by TLC or HPLC analysis) and no racemization occurs during this step. Next, is the treatment of the reaction products with CPD-Y. The solubility of substrates is an important consideration. We have tried various combinations of 50 mM-HEPES, pH 7.5, buffer and methanol, DMSO and DMF. The best one was 20% by volume DMSO in the buffer. Under these conditions the substrates are sufficiently soluble and the enzyme activity is still preserved. The enzymatic hydrolysate can be directly analyzed by HPLC without the necessity of isolating DNP-derivatives. The kinetics of hydrolysis can be followed by this method since an ester and the corresponding acid are eluted at different times.

Until now, CPD-Y hydrolysis has only been applied to artificial mixtures of five amino acids, namely, Ala, Asp, Glu, Met and Phe containing 20%, 10%, 5% and 0%, respectively, of D-isomers. In all cases we obtained good HPLC separation of D-esters from L-acids and detection of less than one nanomole of D-esters could be achieved. However, it is a limitation of the present method that it only can be used to measure the extent of racemization of those amino acids which after conversion to DNP-amino acid methyl esters will be hydrolyzed by CPD-Y.

ACKNOWLEDGEMENTS

We thank Carlsberg Biotechnology Ltd. for the samples of CPD-Y and subtilisin A. Ms. IRENE SIMONSEN is greatfully acknowledged for excellent technical assistance. P. MARFEY thanks Carlsberg Laboratory for financial support.

REFERENCES

1. BUNJAPAMAI, S., R.R. MAHONEY & I.S. FAGERSON: Determination of D-amino acids in some processed foods and effect of racemization on *in vitro* digestibility of casein. J. Food Sci. 47, 1229-1234 (1982)
2. FISHER, E.: Untersuchungen über Aminosären, Polypeptide und Proteine, Berlin, I (1906) and II (1923)
3. JOHANSEN, J.T., K. BREDDAM & M. OTTESEN: Isolation of carboxypeptidase Y by affinity chromatography. Carlsberg Res. Commun. 41, 1-14 (1976)
4. FRIEDMAN, M., J.C. ZAHNLEY & P. MASTERS: Relationship between *in vitro* digestibility of casein and its content of lysinoalanine and D-amino acids. J. Food Sci. 46, 127-131 (1981)
5. SANGER, F.: The free amino groups of insulin. Biochem. J. 39, 507-515 (1945)
6. SOL, B.S.: Factors affecting the extent of racemization of peptide and protein bound amino acids: Positioning effect and kinetic studies. M.S. Thesis. Utah State University. Logan UT, (1978)
7. SVENDSEN, I.: Chemical modification of the subtilisins with special reference to the binding of large substrates. A review. Carlsberg Res. Commun. 41, 237-291 (1976)
8. WIDMER, F., K. BREDDAM & J.T. JOHANSEN: Carboxypeptidase Y catalyzed peptide synthesis using amino acid alkyl esters as amine components. Carlsberg Res. Commun. 45, 453-463 (1980)

Accepted by:
H. KLENOW, E. LUND and S.O. ANDERSEN