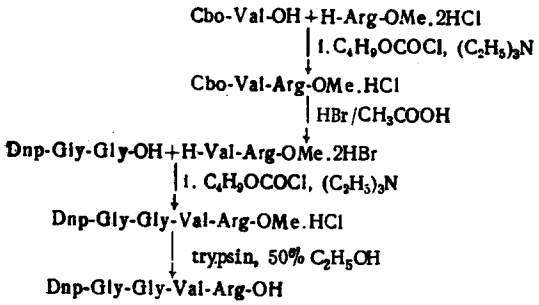
DETERMINATION OF THE ACTIVITY OF NEUTRAL PROTEINASES WITH RESPECT TO THE HOMOGENEOUS SUBSTRATE 2,4-DINITROPHENYLGLYCYLGLYCYL-L-VALYL-L-ARGININE

L. A. Lyubinskaya, L. V. Lastovetskaya,
G.V. Shekhvatova, T. I. Vaganova,
and V. M. Stepanov

UDC 577.156

The most convenient substrates for the determination of the proteolytic activity of enzymes are chromogenic substrates the cleavage of which leads to the appearance of a color or a characteristic color change. We have previously proposed the tripeptide 2,4-DNP-glycylglycyl-L-arginine as the substrate for determining the activity of a number of carboxy-peptidases [1, 2]. In the present work, the possibility is shown of constructing an analogous chromophoric substrate for neutral proteinases – metal-containing enzymes cleaving peptide bonds formed by the amino groups of hydrophobic amino acids. We have synthesized 2,4-DNP-glycylglycyl-L-valyl-L-arginine, which has proved to be a convenient substrate for determining the activity of neutral proteinases – thermolysin and the neutral proteinase from Bacillus subtilis. Both these enzymes cleave the glycyl–valyl bond in this peptide, leading to the liberation of the dinitrophenylated dipeptide DNP-glycylglycine, which differs substantially in its physicochemical properties from the substrate and can be separated from the latter quantitatively by extraction with a nonpolar solvent. The spectrophotometric determination of DNP-glycylglycine presents no difficulties. We synthesized the DNP-glycylglycyl-L-valyl-L-arginine by the following method:



The methyl ester of benzyloxycarbonyl-L-valyl-L-arginine was obtained by coupling benzyloxycarbonyl-L-valine and the hydrochloride of the methyl ester of L-arginine by the mixed anhydride method. To purify the compound we used repeated extraction of an acidic aqueous solution with ether and n-butanol. The yield of hydrochloride of the methyl ester of benzyloxycarbonyl-L-valyl-L-arginine was 90%. The removal of the benzyloxycarbonyl group with hydrogen bromide in glacial acetic acid gave the hydrobromide of the methyl ester of L-valyl-L-arginine with a yield of 98%.

To obtain the methyl ester of DNP-glycylglycyl-L-valyl-L-arginine, the mixed anhydride obtained from DNP-glycylglycine and isobutyl chloroformate was coupled with the methyl ester of L-valyl-L-arginine. Subsequent extraction of the acidic aqueous solution with ether and n-butanol yielded the hydrochloride of the methyl ester of DNP-glycylglycyl-L-valyl-L-arginine in 75% yield. For its saponification we used hydrolysis with trypsin in 50% ethanol at pH 7.8. The yield of DNP-glycylglycyl-L-valyl-L-arginine was 85%. DNP-glycylglyisoleucyl-L-arginine, which is also a substrate for neutral proteinases, can be obtained similarly.

All-Union Scientific-Research Institute of the Genetics and Breeding of Industrial Microorganisms, Moscow. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 75-80, January-February, 1976. Original article submitted December 26, 1974.

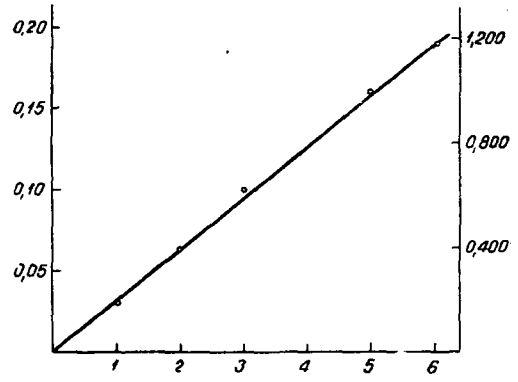


Fig. 1. Cleavage of DNP-glycylglycyl-L-valyl-L-arginine by a preparation of thermolysin (Merck). The amount of thermolysin in the sample (μg) has been plotted along the axis of abscissas, the amount of DNP-glycylglycine (μmole) along the left-hand axis of ordinates, and the optical density at 360 nm along the right-hand axis of ordinates.

Paper electrophoresis at pH 5.6 showed that thermolysin and the neutral proteinase from Bacillus subtilis hydrolyzed DNP-glycylglycyl-L-valyl-L-arginine with the formation of the DNP-glycylglycine and valyl-arginine, which corresponds to the specificity of neutral proteinases. The DNP-glycylglycine formed can be separated quantitatively from the uncleaved substrate by extraction with an organic solvent and be determined colorimetrically at 360 nm.

In the hydrolysis of the substrate by thermolysin at pH 7.0 and 37°C, a linear relationship is observed between the amount of DNP-glycylglycine formed and the amount of enzyme in the sample (1-6 μg; Fig. 1). A linear relationship is also observed between the amount of DNP-glycylglycine formed and the amount of protosubtilin in a sample (50-400 μg; Fig. 2).

Thus, the method permits the quantitative determination of neutral proteinases. However, the following limitations of the method must be pointed out. An acid carboxypeptidase produced, in particular, by some fungi and yeasts may split off arginine from DNP-glycylglycyl-L-valyl-L-arginine. The DNP-glycylglycyl-L-valine formed by this process is extracted by organic solvents like DNP-glycylglycine. Consequently, the presence of acid carboxypeptidases in a complex enzyme preparation may interfere with the determination of the neutral proteinases by the method described, and it is therefore desirable to make sure of the absence of acid carboxypeptideases from the preparation analyzed. The presence of free arginine in the products of hydrolysis of the substrate, which can be established by means of paper electrophoresis or amino-acid analysis, serves as a definite proof of the presence of an acid carboxypeptidase.

On the other hand, complete inhibition of the hydrolysis of the substrate by 0.01 M EDTA is characteristic for neutral proteinases [3] while under these conditions an acid carboxypeptidase retains its activity completely. Inhibition of the reaction by EDTA can be used to check the absence of an acid carboxypeptidase from the preparation. Thus, we have found that the products of the hydrolysis of DNP-glycylglycyl-L-valine-L-arginine by protosubtilin contain no arginine and the activity of this preparation is completely suppressed by 0.01 M EDTA. It follows from this that the protosubtilin does not contain an acid carboxypeptidase.

By using the method described above, we have determined the activity of the neutral proteinase in a number of preparations of protosubtilin and have compared it with the protease activity of these preparations calculated from the cleavage of hemoglobin. The activities of the protosubtilin preparations with respect to casein were determined in the Vilnius factory for enzyme preparations. Table 1 shows the well-known correspondence between the peptidase activity of a neutral proteinase for DNP-glycylglycyl-L-valyl-L-arginine and the total proteinase activity with respect to hemoglobin and casein. This is possibly explained by the presence in the enzyme preparation of small amounts of proteolytic enzymes differing in their specificity from the neutral proteinases.

The method developed is convenient for the routine determination of the activities of neutral proteinases in enzyme preparations. The proposed method is more selective than the determination of the activity for protein substrates. The spectrophotometric method of determining neutral proteinases from the hydrolysis of

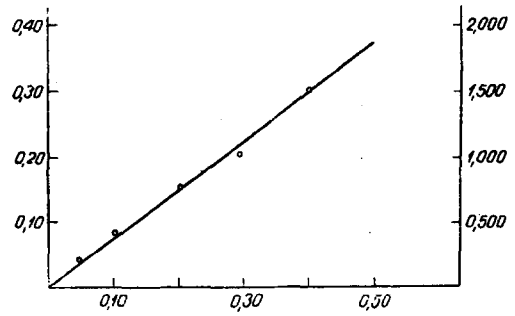


Fig. 2. Cleavage of DNP-glycylglycyl-L-valyl-L-arginine by a partially purified preparation of protosubtilin (preparation 2). The amount of DNP-glycylglycine (μmole) has been plotted along the left-hand axis of ordinates and the optical density at 360 nm along the right-hand axis of ordinates; the amount of protosubtilin (mg) in the sample has been plotted along the axis of abscissas.

TABLE 1. Peptidase and Protease Activities of Thermolysin in Different Preparations of Protosubtilin

Preparation	1 mg E_{280}	Specific activity for casein, units/g	Specific activity for hemoglobin ($E_{280} \times 3$/mg · min) × 10^2	Specific activity for DNP-Gly-Gly-Val-Arg (μmole of substrate/mg · min) × 10^3
Thermolysin	1,210	—	1270	1200
Protosubtilin 1	0,670	112,0	31,2	25,3
Protosubtilin 2	0,610	86,2	24,0	8,0
Protosubtilin 3	0,800	10,0	4,5	1,5
Protosubtilin 4	—	6,0	1,6	0,5
Protosubtilin 5	0,140	1,5	0,6	0,2
Protosubtilin 6	0,625	14,0	4,0	1,3

amides of furylacryloyldipeptides recommended by Feder [4] is suitable for kinetic investigations of pure preparations and requires apparatus with a high sensitivity. The method that we have proposed permits the use of an ordinary spectrophotometer, since the measured optical density is fairly high.

EXPERIMENTAL

Paper Chromatography and Paper Electrophoresis. Descending paper chromatography was performed on "Filtrak" No. 14 paper in the following solvent systems: 1) butan-1-ol–pyridine–water–acetic acid (30:20:24:6), R_{f_1}; 2) butan-1-ol–acetic acid–water (4:5:1), R_{f_2}; and 3) methyl ethyl ketone–pyridine–water (65:15:20), R_{f_3}.

Electrophoresis was performed on "Leningrad" chromatographic paper of type B (medium) for 30 min at a potential gradient of 27 V/cm. A pyridine acetate buffer, pH 5.6 (8 ml of pyridine, 2 ml of acetic acid, and 990 ml of water) was used.

Hydrochloride of the Methyl Ester of Benzyloxycarboxyl-L-valyl-L-arginine. To a solution of 2.51 g (10 mmole) of benzyloxycarbonyl-L-valine and 25 ml of dry dimethylformamide at −15°C were added 1.4 ml (10 mmole) of triethylamine and, after 10 min, 1.4 ml (10 mmole) of isobutyl chloroformate. After 30 min, this material was mixed with a solution, cooled to −15°C, of the hydrochloride of the methyl ester of L-arginine obtained by the addition of 1.4 ml (10 mmole) of triethylamine to 2.61 g of the dihydrochloride of L-arginine methyl ester in 25 ml of dry dimethylformamide. The resulting mixture was stirred with cooling for 1 h and at 20°C for 1 h and was left overnight at 4°C. The triethylamine hydrochloride formed was filtered off, and the filtrate was evaporated in vacuum. The oil obtained was dissolved in 100 ml of 0.1 N hydrochloric acid and the unchanged benzyloxycarbonyl-L-valine was extracted with ether (5 × 25 ml). The completeness of purification was checked by paper electrophoresis. The methyl ester of benzyloxycarbonyl-L-valyl-L-arginine

was extracted from the acidic aqueous solution with n-butanol (5 × 25 ml). The butanolic extract was washed with 10 ml of water and evaporated in vacuum. The residual oil solidified on being triturated with dry ether. Yield 4.1 g (90%), mp 91-93°C, $[\alpha]_D^{22}$ +21.8° (c 1; C_2H_5OH); R_{f_1} 0.91; R_{f_2} 0.85; l 5.7 cm (to the cathode); $C_{20}H_{31}N_5O_5 \cdot HCl$.

Dihydrobromide of the Methyl Ester of L-Valyl-L-arginine. A solution of 5.0 g (11 mmole) of the methyl ester of benzyloxycarbonyl-L-valyl-arginine in 10 ml of a 40% solution of hydrogen bromide in glacial acetic acid was kept at room temperature for 25 min, and then 80 ml of dry ether was added. The oil that formed was separated by decantation and dissolved in methanol, and the solution was evaporated in vacuum (five to six times). This gave a light yellowish amorphous substance. Yield 4.85 g (98%), $[\alpha]_D^{22}$ +1.0° (c 1; absolute ethanol); R_{f_1} 0.64; R_{f_3} 0.60; l 10.4 cm (to the cathode); $C_{12}H_{25}N_5O_3 \cdot HBr$.

Hydrochloride of the Methyl Ester of DNP-Glycylglycyl-L-valyl-L-arginine. To a solution of 2.24 g (7.5 mmole) of DNP-glycylglycine in 20 ml of dry dimethylformamide at −15°C were added 1.05 ml (7.5 mmole) of triethylamine and, after 10 min, 1.05 ml (7.5 mmole) of isobutyl chloroformate. After 30 min, this material was mixed with a solution, cooled to −15°C, of the hydrobromide of the methyl ester of L-valyl-L-arginine obtained by the addition of 1.05 ml of triethylamine to 3.37 g (7.5 mmole) of the dihydrobromide of the methyl ester of L-valyl-L-arginine in 15 ml of dry dimethylformamide. The resulting mixture was stirred with cooling for 1 h and at 20°C for 1 h and was left overnight at 4°C. The precipitate of the triethylamide salt was filtered off, and the filtrate was evaporated in vacuum. The residual brownish oil was dissolved in 2.3 liters of 0.1 N hydrochloric acid, the insoluble residue was filtered off, and the filtrate was extracted repeatedly with ether (10 × 400 ml) to eliminate traces of DNP-glycylglycine. The completeness of purification was checked by paper electrophoresis. The methyl ester of DNP-glycylglycyl-L-valyl-L-arginine was extracted from the acidic aqueous solution with n-butanol (6 × 400 ml). The butanolic solution was washed with water (3 × 80 ml) and evaporated in vacuum. The yellow oil obtained solidified on trituration with ether. Yield 3.40 g (75%), mp 119-121°C; $[\alpha]_O^{22}$ − 6° (c 1; DMFA), R_{f_1} 0.92; R_{f_2} 0.88; R_{f_3} 0.91; l 3.8 cm (to the cathode); $C_{22}H_{33}N_9O_9 \cdot HCl \cdot C_4H_9OH$.

DNP-Glycylglycyl-L-valyl-L-arginine. A solution of 2 g (3.3 mmole) of the methyl ester of DNP-glycyl-valyl-L-arginine in 200 ml of 96% ethanol was treated with 8 mg of trypsin in 200 ml of 0.2 M triethylamine carbonate buffer, pH 7.8, and the mixture was kept at 37°C for 3 h. Then the hydrolyzate was separated in vacuum and was reprecipitated from methanol with ether. Yield 1.55 g (85%), mp 172-174°C, $[\alpha]_D^{22}$ − 24.0° (c 1; DMFA), R_{f_1} 0.74; R_{f_2} 0.87, R_{f_3} 0.78; l 1.1 cm (to the cathode); $C_{21}H_{31}N_9O_9 \cdot CH_3OH$.

Enzymes. The thermolysin (neutral proteinase from Bacillus thermoproteolyticus) was a Merck (GFR) product. The enzyme preparations of the neutral proteinase of Bacillus subtilis (protosubtilin) with different degrees of purification were obtained from the Vilnius factory for enzyme preparations.

Determination of the Activity of the Neutral Proteinases. A 0.2 μM solution of DNP-glycylglycyl-L-valyl-L-arginine in 0.05 M tris-HCl buffer, pH 7.0, containing 0.01 M $CaCl_2$, was prepared. Since the substrate is hygroscopic, its concentration was checked from the absorption of the solution at 360 nm, taking the molar extinction of the DNP-amino group as 15,000.

To 1 ml of a solution of the neutral proteinase in 0.05 M tris-HCl buffer, pH 7.0, containing 0.01 M calcium chloride was added 4 ml of a solution of the substrate. The mixture was incubated at 37°C for 30 min, after which 0.2 ml of 1 N hydrochloric acid was added to pH 1-2 and it was extracted with 5 ml of ethyl acetate containing 10% of ethanol. The upper layer was extracted with 4 ml of a 1% solution of sodium bicarbonate. The lower layer obtained in this process was transferred to a 1-cm quartz cell and its optical density was determined at 360 nm. The concentration of the enzyme was selected so that the optical density of the measured solution did not exceed 1. A control experiment was performed under the same conditions with the addition of the enzyme to a solution of the substrate acidified with hydrochloric acid.

The protease activity with respect to hemoglobin was determined by a published method [5] at pH 7.0 in the presence of 0.01 M calcium chloride. The activity of the neutral proteinase was judged from the absorption at 280 nm of a trichloroacetic acid filtrate of a hemoglobin hydrolyzate obtained under standard conditions in 1 min.

SUMMARY

1. A chromogenic specific substrate for neutral proteinases − 2,4-dinitrophenylglycylglycyl-L-valyl-L-arginine − has been synthesized.

2. A method has been developed for determining the activity of neutral proteinases in enzyme preparations

with the use of this substrate which is based on the spectrophotometric determination of DNP-glycylglycine formed on the cleavage of the glycyl–valyl bond in the substrate.

3. The applicability of the method to the determination of thermolysin and of the neutral proteinase from Bacillus subtilis has been shown.

LITERATURE CITED

1. L. A. Lyubinskaya, T. I. Vaganova, T. S. Paskhina, and V. M. Stepanov, Biokhimiya, 38, 790 (1973).
2. L. V. Lastovetskaya, T. I. Vaganova, and V. M. Stepanov, Khim. Prirodn. Soedin., 819 (1974).
3. J. D. McConn. D. Tsuru, and K. T. Yasunobu, J. Biol. Chem., 239, 3706 (1964).
4. J. Feder, Biochem. Biophys. Res. Commun., 32, 326 (1968).
5. J. Northrop, M. Kunitz, and R. Herriott, Crystalline Enzymes, Columbia University Press, New York (1948).

INVESTIGATION OF THE DIOXANE LIGNIN OF THE COTTON PLANT

N. A. Veksler, L. S. Smirnova, and Kh. A. Abduazimov

UDC 547.621:032.11

Continuing a study of the dioxane lignin (DLA) from the stems of cotton plants in various vegetation periods, we have isolated DLA-I (early stage, two-seed leaves) and DLA-II (flowering stage) by a method described previously [1]. The lignins were purified by Freudenberg's method [2].

The preparations obtained are light brownish amorphous powders readily soluble in the usual solvents for lignins and in water.

After purification, the DLA-I contained 6% and the DLA-II 6.4% of carbohydrates as determined by Bertrand's method [3]. These carbohydrates are obviously included in the composition of the lignin in the form of a lignin–carbohydrate complex. In the subsequent calculations, a correction was introduced for the carbohydrate content.

The UV spectrum of each preparation has the maximum at 280 nm that is characteristic for lignins. It differs from the UV spectrum of the dioxane lignin of a later vegetation period DLA-III by the presence of a shoulder in the 310-360 nm region. The IR spectra of the preparations showed the bands of a benzene ring with substituents (1610-1620, 1525, 1450-1455 cm^{-1}) and of carbonyl (1720 cm^{-1}), hydroxy (3400 cm^{-1}), and ether (1260, 1120-1125, 1075-1090 cm^{-1}) groups.

The gel chromatography of the DLA-I and DLA-II in a column containing Sephadex G-75 equilibrated with dimethyl sulfoxide showed (Fig. 1) that the DLA-I is monomodal and the DLA-II bimodal, the amount of low-molecular-weight fraction in the latter being very small. In a comparison with a gel chromatogram of the dioxane lignin isolated from the ripe stems of the cotton plant (DLA-III) it can be seen that the latter contains a larger amount of the low-molecular-weight fraction than the DLA-II. The ratio of the low-molecular-weight to the high-molecular-weight fraction for DLA-III is 1 : 2.5 and for DLA-II 1 : 6. In these lignins, the high-molecular-weight fraction is present in the greatest amount. The molecular weights calculated by using the coefficients given by Alekseev et al., [4] are practically the same for all three samples (22,000-23,500). Consequently, all the DLAs of the stem of the cotton plant are of high molecular weight and as the plant develops the lignin becomes less homogeneous, since the amount of low-molecular-weight fraction in it increases.

Institute of the Chemistry of Plant Substances, Academy of Sciences of the Uzbek SSR, Tashkent.

Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 80-83, January-February, 1976. Original article submitted January 10, 1975.