A CHROMOGENIC SUBSTRATE OF α-CHYMOTRYPSIN — THE p-NITROANILIDE OF L-PYROGLUTAMYL-L-PHENYLALANINE

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Chymotrypsin hydrolyzes the p-nitroanilide of pyroglutamyl-L-phenylalanine with the splitting out of p-nitroaniline, which is determined spectrophotometrically from its absorption at 410 nm. The pyroglutamic acid residue increases the solubility of this substrate in water as compared with the p-nitroanilide of N-(3-carboxypropionyl)-L-phenylalanine that is usually used [1], which permits the determination of the activity of the enzyme in the presence of small amounts of organic solvent and the avoidance of the inhibiting action of the latter. In the hydrolysis of the substrate, a linear dependence of the optical density at 410 nm on the amount of chymotrypsin added is observed in the range of 10-100 μg. The specific activity of α-chymotrypsin measured from the hydrolysis of the p-nitroanilide of pyroglutamyl-L-phenylalanine is 0.035 μmole of substrate/min/mg of enzyme, which exceeds by an order of magnitude the specific activity determined with respect to the p-nitroanilide of N-(3-carboxypropionyl)-L-phenylalanine.

To synthesize the p-nitroanilide of pyroglutamyl-L-phenylalanine, 0.494 g (24 mmole) of dicyclohexylcarbodiimide was added to 0.309 g (24 mmole) of pyroglutamic acid and 0.345 g (30 mmole) of N-hydroxysuccinimide in 8 ml of dry ethyl acetate, with stirring and cooling to 0°C. The reaction mixture was stirred at 0°C for 8 h and was left at 20°C for 12 h. The dicyclohexylurea was filtered off, and the filtrate was evaporated in vacuum to dryness. The residue was dissolved in 8 ml of dry tetrahydrofuran (THF), and, with stirring and cooling, a solution of 0.684 g (24 mmole) of L-phenylalanine p-nitroanilide in 7 ml of dry THF was added, and the mixture was stirred at 0°C for 2 h and was left at 20°C for 48 h. The precipitate of the p-nitroanilide of pyroglutamyl-L-phenylalanine that deposited was filtered off, washed on the filter with THF and ether, and dried in a vacuum desiccator over P_2O_5. Yield 0.7 g (74%), mp 265-268°C (decomp.) $[\alpha]_D^{20}$ + 60° (c 0.3; 70% dimethylformamide). Elementary analysis: $C_{20}H_{20}O_5N_4 \cdot 1/2\ H_2O$. Found, %: C 59.50; H 5.03; N 14.34. Calculated %: C 59.25; H 5.18; N 13.82. R_f 0.75 (TLC on Silufol plates in the pyridine—n-butanol—water—acetic acid (20 : 30 : 12 : 6) system).

We then determined the activity of α-chymotrypsin. To 30 μl of a solution of the substrate in absolute dimethylformamide (20 mg/ml) was added 2 ml of Tris-HCl buffer, pH 7.65, containing 66 mM $CaCl_2$. The mixture was kept at 37°C for 2 min, and then a solution of chymotrypsin was added and incubation was carried out at 37°C for the time necessary for the appearance of a yellow coloration (usually 8-10 min). The reaction was stopped by the addition of 1 ml of 30% acetic acid, the time was noted on a stopwatch, and the optical density at 410 nm was measured. To prepare the control solution, the same reaction mixture was incubated at 37°C and the chymotrypsin was added after 30% acetic acid. The specific activity was calculated by using the relation $E_M^{410} = 8900$.

LITERATURE CITED

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