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REGENERATION OF AMINO COMPOUNDS FROM THE 2,4,6-TRINITROPHENYL DERIVATIVES BY TREATMENT WITH HYDRAZINE

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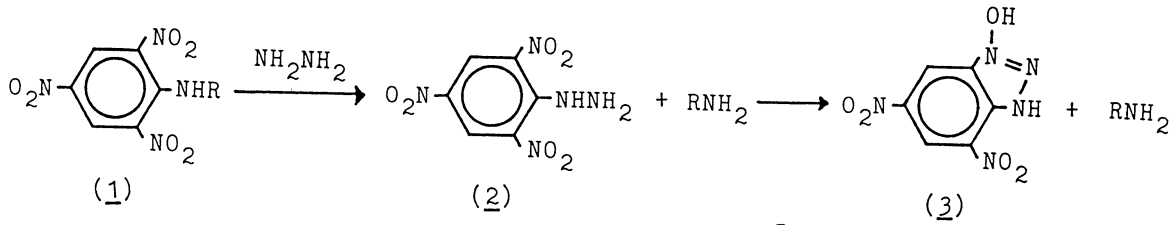
2,4,6-Trinitrophenylalanine gave free alanine and 1-hydroxy-4,6-dinitrobenzotriazole by treating with aqueous solution of hydrazine at pH 7–9 and 30 °C. Applying the reaction to partially trinitrophenylated derivative of lima bean trypsin inhibitor, the fully active protein could be regenerated almost quantitatively.

The trinitrophenyl(TNP)ation with sodium 2,4,6-trinitrobenzenesulfonate (TBS)[1] is widely used for spectrophotometric assay of amines,[2] amino acids,[3,4] and amino groups in proteins.[5] As the reaction proceeds preferentially on amino groups under a mild condition, TBS has been used for the modification of proteins[6–9] and intact cells[10,11] to elucidate the amino groups participating in their biological functions. Provided that the TNP group so introduced is easily removed under a mild condition, the reaction will be more widely used for the reversible modification of amino groups. The present letter describes the regeneration of amino groups from the TNP derivatives with hydrazine according to the following scheme:



(1) (2) (3)

On the incubation of an aqueous solution of 1×10^{-3} M TNP-alanine (-Ala) (1) with 5×10^{-2} M (1 M=1 mol dm^{-3}) hydrazine hydrochloride at pH 8.6 (adjusted with sodium hydroxide) and 30 °C, an irreversible change with the increasing reaction time was observed on the UV-spectrum as shown in Fig. 1 (A). TLC of the reaction mixture indicated the conversion of TNP-Ala to free Ala and TNP-hydrazine (2) and the successive change of (2) to a yellow compound "X". The Rf value on silica plate developed with a mixture of 2-propanol - benzene - M/100 hydrochloric acid (55:30:15, v/v) was 0.60 for TNP-Ala, 0.08 for Ala, 0.95 for TNP-hydrazine, and 0.55

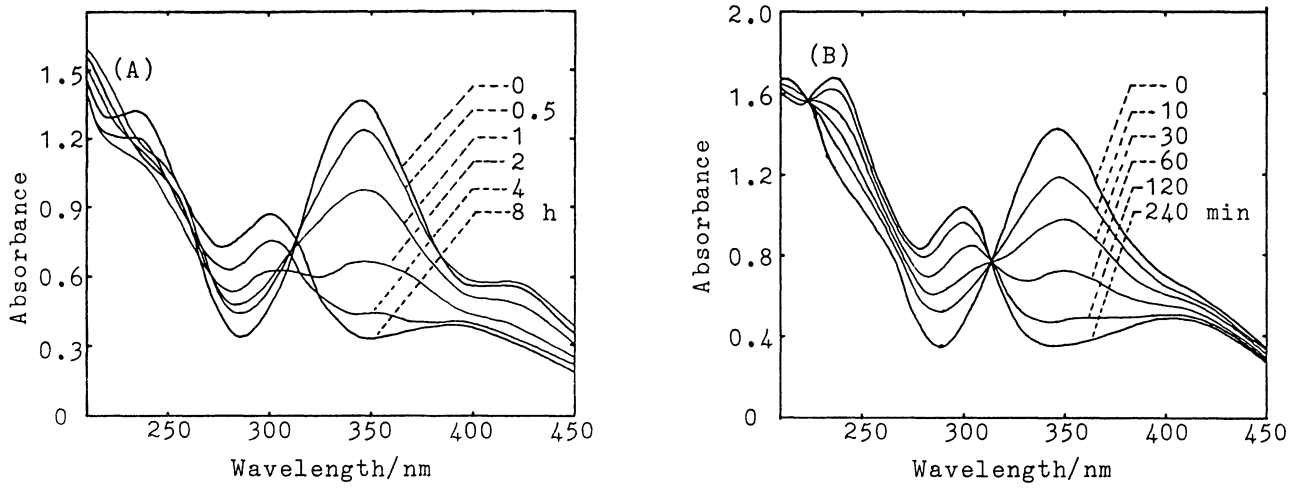


Fig. 1. Spectral changes of TNP derivatives at 30 °C. (A), $1x10^{-3}$ M TNP-Ala treated with $5x10^{-2}$ M hydrazine at pH 8.6, observed after 10 times dilution; (B), $1.2x10^{-4}$ M TNP-hydrazine at pH 7.2.

for "X". When authentic TNP-hydrazine[12] was incubated in M/10 NaH_2PO_4 –M/20 $Na_2B_4O_7$ buffer containing 10% methanol, pH 7.2 at 30 °C, a spectral change was also observed, affording a yellow product chromatographically indistinguishable from "X". In this case, two isosbestic points were observed at 223 and 314 nm (Fig. 1 (B)), differing from the reaction of TNP-Ala with hydrazine (Fig. 1 (A)). These results indicate that TNP-hydrazine is an intermediate in the formation of "X" from TNP-Ala as shown in the above scheme. The yellow products from TNP-Ala treated with hydrazine and from TNP-hydrazine were isolated in yield of more than 80% as fine yellow needles with mp 200–201 °C from the benzene extract of acidified reaction mixture, respectively. Their IR spectra (400–4000 cm^{-1}) were essentially the same. The analytical data of "X" from TNP-Ala were as follows. Found: C, 28.9; H, 2.0; N, 29.1%. Calcd for $C_6H_3N_5O_5$ H_2O: C, 29.1; H, 2.1; N, 28.8%. IR ν_{max} (cm^{-1}, KBr): 3400 (NH and/or OH), 3100 (arom. CH), 1640 (-N=N-), 1615, 1502, 1437 (arom. ring), 1540, 1340 ($-NO_2$), and 1282 ($-N^+O^-$). ^{1}H-NMR δ (DMSO-d_6): 9.06 (1H, d, J=2 Hz) and 8.86 (1H, d, J=2 Hz). MS (m/e): 225 (M^+). Then "X" was deduced to be 1-hydroxy-4,6-dinitrobenzotriazole. The pKa of "X" was spectrophotometrically determined to be 1.6 (Fig. 2). The value is reasonable for the deduced structure, as those of 1-hydroxybenzotriazole and the 4- and the 6-nitro derivatives have been reproted to be 7.9, 3.4, and 2.8, respectively.[13] As well known, 2-nitrophenylhydrazine gives 1-hydroxybenzotriazole by refluxing the ethanol solution.[14] In the present case, a similar cyclization occurred under a milder condition (pH 7–9 and 30 °C), presumably due to the presence of the two additional nitro groups at

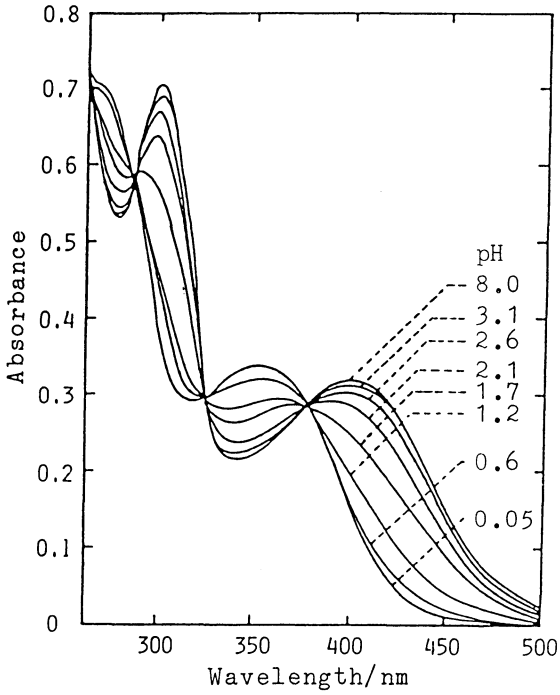


Fig. 2. pH dependence of the absorption spectrum of 1-hydroxy-4,6-dinitrobenzotriazole.

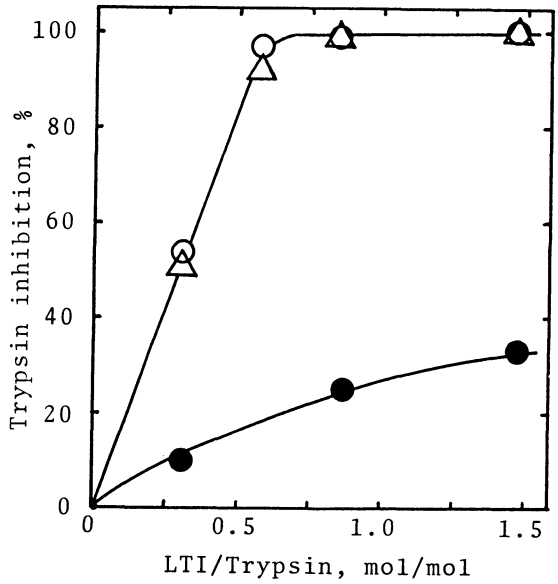


Fig. 3. Inhibitory activity of intact (○), TNPated (●) and recovered (△) LTI.

the 4 and 6 positions.

The reaction was applied to lima bean trypsin inhibitor (LTI) in order to regenerate a biologically active protein from the TNP derivative. As already reported by Feeney et al.,[15] amino groups of the bivalent inhibitor are essential for the formation of $(Trypsin)_2$-LTI complex and the TNPation with TBS brings about a marked decrease in the inhibitory activity. LTI (from Sigma Chemical Co., 3 times recrystallized) was TNPated in M/10 NaH_2PO_4-M/20 $Na_2B_4O_7$ buffer pH 9.0 with TBS at molar ratio of 40 (TBS)/1 (LTI). After the incubation at 35 °C for 4 h, the reaction mixture was dialyzed in the buffer (pH 8.0) to remove the excess reagent. From the protein concentration (determined by Biuret method[16]) and the TNP content (spectrophotometrically determined, taking the molar extinction coefficient of N-TNP as 1.3×10^4 $M^{-1}cm^{-1}$ at 346 nm), it was indicated that about 2.5 of 5 amino groups in LTI were TNPated as an average. The inhibitory activity of TNP-LTI (assayed on the tryptic hydrolysis of N^{α}-benzoyl-L-arginine-p-nitroanilide[17]) at various LTI/trypsin ratios indicated a marked decrement of binding affinity to trypsin. On the incubation of 1×10^{-4} M TNP-LTI with 2×10^{-1} M hydrazine at pH 9.0 and 35 °C, a spectral change characteristic of the conversion of N-TNP to 1-hydroxy-4,6-dinitrobenzotriazole was observed. After the completion of the change (10 to 15 h),

the reaction mixture was dialyzed to recover the protein moiety, of which absorbance at λ_{max} 346 nm of N-TNP group was negligible. The measurement of the inhibitory activity indicated that LTI of full activity was regenerated almost quantitatively from the partially TNPated and inactivated derivative by treatment with hydrazine, as shown in Fig. 3.

Several methods have been reported on the regeneration of active proteins from the N-acyl derivatives as follows: acid hydrolysis of N-acetimidino[18] and N-maleyl derivatives[19] and aminolyses of N-trifluoroacetyl derivatives with piperidine[20] and of N-acetoacetyl derivatives with hydrazine[21] or hydroxylamine.[22] As to the N-aryl-proteins, Tamaoki et al.[23] reported the regeneration of Taka-amylase from the partially nitrotroponylated and inactivated derivative with 1–2 M hydrazine at pH 8–9 and 38 °C in yield of 75%, without elucidating the product from nitrotroponyl residues. Our method is more excellent both on the yield and on the reaction conditions. The reversible modification of amino groups with TBS followed by treatment with hydrazine will be valuable for study of amino groups participating in the protein functions.

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