SIMPLE METHODS OF PREPARING NICOTINAMIDE MONONUCLEOTIDE

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1. Introduction

Nicotinamide mononucleotide (NMN) is the functional moiety of the coenzyme $NAD^{\oplus}$, starting material for the preparation of many coenzyme analogs with an altered nonfunctional moiety [1]. NMN is obtained by enzymatic cleavage of the $NAD^{\oplus}$-pyrophosphate bond using $NAD^{\oplus}$-pyrophosphatase (dinucleotide-nucleotido hydrolase, EC 3.6.1.9) [2,3]. Moreover, several chemical methods of synthesizing the compound have been described [4–7].

This paper is to report the preparation of NMN starting with $NAD^{\oplus}$-employing $NAD^{\oplus}$-pyrophosphatase from potatoes bound to a supporting matrix as well as a simple chemical method using 2,3-O-isopropylidine-D-ribofuranosylamine as starting material [8].

2. Materials and methods

2.1. *Materials*

YADH, LADH from horse and $NAD^{\oplus}$ were obtained from Boehringer & Soehne, Mannheim, CNBr Sepharose 4B from Pharmacia, Uppsala, Sweden.

2.2. *General procedures*

The enzymatic activities of $NAD^{\oplus}$-pyrophosphatase solutions from potatoes were measured according to Kornberg and Pricer [2] as well as by measuring the increase in extinction at 260 nm [9] with a Cary 14 spectrophotometer. A third method is to titrate the protons during the hydrolysis of the $NAD^{\oplus}$-pyrophosphate bond, using an Autotitrator Kopenhagen TTT3. Acetate strips and 0.1 M acetate buffer were used for the electrophoresis of the $NAD^{\oplus}$-pyrophosphatase. The protein was detected by staining with Naphthalene Black B. The enzymatic activity was detected by spraying the electropherogramm with 1 mM $NAD^{\oplus}$ in 0.1 M phosphate buffer at pH 7.6; after 30 min at 20°C the strip was sprayed with a solution containing 5 mg YADH and 1 ml ethanol in 100 ml pyrophosphate–glycine–semicarbazide buffer of pH 8.7. In UV light the region of $NAD^{\oplus}$-pyrophosphatase activity shows up as a dark zone. 2.15 mg $NAD^{\oplus}$-pyrophosphatase 189 U/mg were covalently bound to 1 g CNBr Sepharose 4B as described by Axén et al. [10].

2.3. *Isolation of $NAD^{\oplus}$-pyrophosphatase*

The juice of 75 kg potatoes (trade name: Bintje) was collected and adjusted with dilute acetic acid to pH 4.4. Forty litres of juice were obtained and divided in 10 fractions 4 l each. These fractions were cooled to −2°C and cold acetone (−15°C) was added under stirring to a final concentrations of 35% vol., reducing the temperature further to −5°C during the addition. The precipitate was removed by centrifugation at 2000 *g*. In the supernatant the concentration of acetone was increased to 50% vol. and the suspension was centrifuged at 2000 *g*. The precipitates of the 10 fractions were collected and taken up in 2 l of water at 0°C. This solution was dialysed for 18 hr at 0°C against 3 × 10 l 0.1 M phosphate buffer (pH 7.5). To the dialysed solution cold acetone (−15°C) was added to a final concentration of 33% vol. The precipitate was collected by centrifugation (1 hr, 0°C, 2000 *g*), dissolved in 15 ml

0.01 M phosphate buffer pH 6.5 and applied to a CM-Sephadex column (68 × 2 cm, equilibrated with 0.01 M phosphate buffer pH 6.5). To elute, a NaCl gradient was used (2 l of 0.5 M NaCl in 0.01 M phosphate buffer pH 6.5 and 2 litres of 0.01 M phosphate buffer pH 6.5 in the mixing vessel). The enzyme appeared in a volume of 480 ml after 1100 ml had passed the column. The enzyme was precipitated at 0°C by addition of solid ammonium sulphate to a final concentration of 70% saturation. The precipitate was collected by centrifugation, dissolved in 1.2 ml of 0.1 M phosphate buffer pH 6.8 and chromatographed on a Sephadex G 200 column (91 × 1.4 cm, eluant 0.1 M phosphate buffer pH 6.8). 4.5 mg of the enzyme were collected in a volume of 6 ml after 70 ml had passed the column.

2.4. *Synthesis of NMN by enzymatic cleavage of $NAD^{\oplus}$*

2.5 ml of Pyrophosphatase–Sepharose 4B gel (0.6 mg of $NAD^{\oplus}$-pyrophosphatase/ml) were suspended in 0.1 M phosphate buffer pH 7.5 and filled into a column (5 cm × 0.8 cm). From a reservoir 100 ml 10 mM $NAD^{\oplus}$ solution in 0.1 M phosphate buffer pH 7.5 flowed through the column (40 ml/hr) at 25°C. The effluent, which contained no more $NAD^{\oplus}$, was concentrated in vacuo and applied to a Dowex 1 × 8 column (100–200 mesh, formiate form, 100 × 3 cm). NMN was eluted with water and appeared in a volume of 150 ml after 300 ml. The solution was concentrated under reduced pressure at 30°C to 30 ml, applied to a Dowex 50 W × 8 column (200–400 mesh, H^+form, 3 × 150 cm) and again eluted with water; after 9 litres had passed the column NMN was eluted in 380 ml. The solution was concentrated in vacuo to 5 ml and the nucleotide was precipitated by addition of cold acetone. The yield was 270 mg.

2.5. *Chemical synthesis of NMN*

48.3 g of 2,3-O-Isopropylidene-D-ribofuranosylamine toluene-*p*-sulphonate [8] were dissolved in 300 ml dry methanol and added to 100 ml Dowex 1 × 8 OH^- form in 300 ml methanol to remove the anion. After removal of the ion exchange resin by filtration and of the solvent (in vacuo at 30°C) a syrupy liquid remained to which 35.8 g of *N*1-(2,4-dinitrophenyl)-3-carbamoyl-pyridinium chloride (110 mMol) in 240 ml methanol were added. The mixture was allowed to stand over night. The methanol solution was filtered off from the dinitroaniline, which was washed with a little methanol. The combined material was concentrated in vacuo at 30°C. To this concentrate ether was added, the precipitate was washed in ether and then taken up in 200 ml methanol. To the solution 70 g dry Dowex 1 × 8 HCO_3^- form were added for 5 min and then removed by filtration and washed with methanol. The solvent was evaporated under reduced pressure at 30°C, ether added to the syrup and by rubbing with a glass rod crystallization was started. The yield was 34.9 g crude nicotinamide-2,3-isopropylidene-ribofuranoside.

25 g of nicotinamide-2,3-isopropylidene-ribofuranoside were added to 250 g meta phosphoric acid [11] and allowed to stand for 2 hr at 35°C, occasionally stirred. The reaction mixture was added to 800 ml 1 N HCl under cooling and kept for 22 hr at 20°C. The reaction mixture was adjusted to pH 4.5 using Dowex 1 × 8 HCO_3^- form (20–50 mesh). The ion exchange resin was filtered off and washed with 3 l water. Filtrate and washings were concentrated to 50 ml at 30°C in vacuo, applied to a Dowex 50 W × 8 column (200–400 mesh, H^+ form, 65 × 4 cm) and eluted with water. β-NMN appeared after 6 l of effluent in 2.6 l. 5 ml 1 N HCl were added to the solution which was concentrated to a syrup in vacuo at 30°C. 6.1 g colorless product was obtained after precipitation with acetone.

3. Results

Using fractional acetone precipitations, CM-Sephadex C 50 ion exchange chromatography and Sephadex G-200 chromatography the dinucleotide–nucleotido hydrolase from potatoes was obtained with a specific activity of 189 U/mg (table 1).

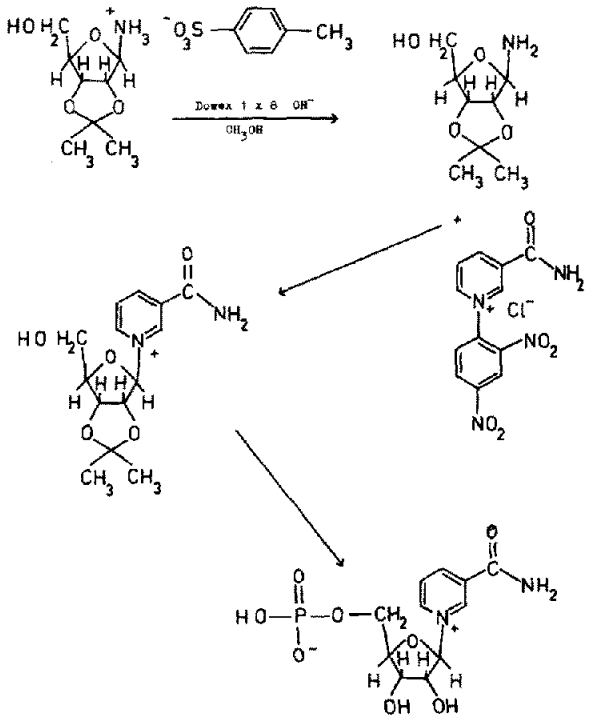
Electrophoresis of the enzyme preparation showed a single protein band. The absorption and fluorescence spectra are those typical for tryptophane containing proteins ($E^{1\%}_{1\,cm} = 12, \lambda_{max} = 280$ nm). In presence of AMP the tryptophane fluorescence is quenched. The fluorescence quenching is used to determine the K_D of the complex to be 3 mM [12]. This is also the value of the inhibitor constant in the

Table 1
Summary of the purification procedures

Step	Enzyme (Units)	Protein (mg)	Specific Activity (U/mg)	Yield %
Crude extract	16820	252000	0.065	100
1. Acetone precipitation	11700	46600	0.25	70
2. Acetone precipitation	6840	910	7.3	42
Cm-Sephadex C 50 chromatography	1700	22	75	10
Sephadex G-200 gel filtration	850	4.5	189	5.1

competitive inhibition of the $NAD^{\oplus}$ cleavage by AMP. The enzyme was bound covalently to CNBr activated Sepharose 4B [10] without significant loss of enzymatic activity. 1.5 mg of the immobilized enzyme split 1 mMol $NAD^{\oplus}$ completely in 2.5 hr. Column chromatography of the nucleotide mixture using Dowex 1 X 8 formiate form and Dowex 50 W X 8 H^+ form results in complete removal of AMP and off the buffer salts [13].

2,3-O-Isopropylidene-D-ribofuranosylamine [8] reacts with *N*1-(2,4-dinitrophenyl)-3-carbamoyl-pyridinium chloride to give the protected riboside (c.f. formula). After treatment with Dowex



1 X 8 HCO_3^- form and removal of the solvent the 5'-position is esterified with meta phosphoric acid at 35°C [11]. The polyphosphates were hydrolysed and the isopropylidene group was split off with dilute hydrochloric acid. The reaction mixture was neutralised with Dowex 1 X 8 HCO_3^- form. The separation of β-NMN from isomers and degradation products was achieved by chromatography with Dowex 50 H^+ form. The yield was 25%. NMN was identified by its enzymatic and physicochemical properties, which were identical with those of NMN prepared from $NAD^{\oplus}$ [14]. The product of condensation of the chemically prepared β-NMN with AMP was a dinucleotide showing the characteristics of β-$NAD^{\oplus}$ [15].

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