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Assay of nicotinamide deamidase activity using high-performance liquid chromatography

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Abstract

A rapid, simple and reproducible method has been developed for the determination of nicotinamide deamidase activity using high-performance liquid chromatography (HPLC). Nicotinic acid (NA) liberated from nicotinamide (NAA) after a 15-min enzyme reaction was determined directly by HPLC without further separation steps. Both NA, the product, and NAA, the substrate were separated by reversed-phase ion-pair isocratic chromatography and detected at 261 nm. The present method could be applied to the measurement of deamidase activity in crude cell extracts prepared from several bacterial strains. The Michaelis constant of nicotinamide deamidase in *Enterobacter agglomerans* was 36 μ*M* for NAA. This method is useful for the measurement of nicotinamide deamidase from various sources.

Keywords: *Enterobacter agglomerans*; Nicotinamide deamidase; Enzymes

1. Introduction

Nicotinamide deamidase (EC 3.5.1.19) is a hydrolase which catalyzes the deamidation of nicotinamide (NAA) to nicotinic acid (NA) and ammonia.

NA and NAA are used in various processed foodstuffs as an enriching or a color fixing agent. In Japan, however, the use of these are prohibited in meat. We previously reported [1,2] that in spite of these compounds not being added, NA was detected together with NAA from several meat samples stored for a few days at 10°C, although the former is usually absent in meat while the latter is present endogenously at levels of 20–60 μg/g. We further indicated that the appearance of NA in the meat was caused by contaminating bacteria and that several bacteria isolated from meat possessed the ability for converting NAA to NA.

It is necessary to confirm that NA is enzymatically formed from NAA by nicotinamide deamidase in these bacteria, and whether bacteria isolated from meat possess deamidase activity. We attempted to develop a simple and accurate method for the determination of nicotinamide deamidase activity.

Several assay methods for nicotinamide deamidase activity have been reported, namely the methods using a radioactive substrate [3–7] and colorimetric methods for liberated ammonia [8,9]. Since the former methods are based on the measurement of radioactively labeled NA which is separated by

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column chromatography [3], paper chromatography [4–6] or thin layer chromatography [7], they are all time-consuming. Meanwhile, the colorimetric methods have poor specificity for the assay of deamidase activity in a biological sample, because they do not directly determine the NA liberated from NAA. Recently high-performance liquid chromatography (HPLC) has been used for the assay of various enzyme activities, because it is able to simultaneously monitor the amount of substrate and product in the reaction mixture. A method using HPLC for deamidase activity in yeast was reported by Yan et al. [10]. However, their method was not applicable to our enzyme from bacteria. Therefore, we investigated a simple, rapid and accurate assay method to determine nicotinamide deamidase activity by HPLC and performed a kinetic study on the partially purified enzyme from *Enterobacter agglomerans* using this method.

2. Experimental

2.1. Chemicals and reagents

NA, NAA and tetrabutylammonium bromide (TBA) were obtained from Tokyo Chemical Industry (Tokyo, Japan). Dithiothreitol (DTT) was purchased from Wako Pure Chemical Industries Tokyo, Japan), and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Pefabloc SC) was from E. Merck (Darmstadt, Germany). Ultrafiltration membrane (STAT-UF) was obtained from Millipore (Tokyo, Japan). All other chemicals were reagent grade.

2.2. Microorganisms and culture

Several strains isolated from meat, described in our previous paper [2], which possessed deamidation activity were used in this study. The strains were inoculated into nutrient broth (Oxoid) and preincubated at 35°C overnight. One milliliter of each suspension was added to Erlenmeyer flasks containing 200 ml of nutrient broth. The flasks were incubated on a rotary shaker (130 rpm) at 35°C for 24 h. The cells were harvested by a centrifugation at 15 000 *g* for 10 min, washed twice with 0.9% sodium chloride solution and stored at −20°C until use.

2.3. Preparation of cell extracts

Extracts for activity measurements were prepared from bacteria isolated from meat. The frozen cell paste, about 1.5 g except for 16 g of B-1 strain, was thawed and suspended in 4.5 ml (B-1 strain, 48 ml) of 20 m*M* phosphate buffer (pH 7.2) containing 1 m*M* DTT, 0.1 m*M* Pefabloc SC, before being disrupted with aluminum oxide in a chilled mortar. The disrupted cell suspension was centrifuged at 20 000 *g* for 10 min to remove cell debris and unbroken cells. The resulting supernatant was used as the crude extract.

2.4. Partial purification of nicotinamide deamidase

Partial purification for the B-1 strain (identified as *Enterobacter agglomerans* [2]) was performed. Solid ammonium sulfate was added to the crude extract by stirring until the solution reached 35% saturation (209 mg/ml). The mixture was allowed to stand at 4°C for 30 min, and then centrifuged at 20 000 *g* for 10 min. The supernatant was next brought to 70% saturation with ammonium sulfate (238 mg/ml). The precipitate was collected by centrifugation and dissolved in 20 ml of buffer A (5 m*M* sodium phosphate (pH 7.2), 5% glycerol and 1 m*M* DTT) with 1 *M* ammonium sulfate. The mixture was applied to a Phenyl Sepharose CL-4B column (Pharmacia) (2×5 cm) equilibrated with the same buffer (pH 7.2). The adsorbed proteins were eluted stepwise from the column with 20 ml of buffer A containing 1 *M*, 0.5 *M*, 0.1 *M* and 0 *M* ammonium sulfate successively. The eluent with no ammonium sulfate contained the deamidase activity which was diluted with an equivalent volume of buffer A containing 2 *M* ammonium sulfate to obtain a 1 *M* ammonium sulfate solution and rechromatographed on the same column (1×1 cm). The active fraction was concentrated with an ultrafiltration membrane.

2.5. Measurement of activity

Nicotinamide deamidase activity was assayed by measuring NA in a reaction mixture (total volume of

1 ml) containing 200 μl of 0.5 *M* sodium phosphate buffer (pH 7.5), 100 μl of 10 m*M* NAA as substrate, 600 μl of distilled water and 100 μl of enzyme solution. The reaction was started by addition of the substrate. Following incubation of the mixture at 37°C for 15 min, the reaction was terminated by addition of 100 μl of 1 *M* acetic acid, and the mixture was heated for 2 min in boiling water. After cooling, the mixture was filtered through Millipore membrane filter (Milex, pore size 0.45 μm), and aliquots of 10 μl were injected into the HPLC system. One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of NA per min under the assay conditions described above.

2.6. HPLC conditions

A Hewlett-Packard 1050 Series high-performance liquid chromatograph including a Model HP79852A pump, and a Model HP79855A autosampler was used together with a variable-wavelength detector (Jasco UV-100 IV). The column used was an L-column ODS (particle size 5 μm, 150 mm×4.6 mm I.D.) (Chemical Inspection and Testing Institute, Japan). The mobile phase consisted of methanol–acetate buffer (adjusted with acetic acid to pH 5) containing 0.1 *M* sodium acetate and 0.01 *M* TBA as an ion-paring agent (15:80, v/v), which was delivered at a flow-rate of 1.2 ml/min at room temperature. The column eluate was monitored at 261 nm.

2.7. Protein measurement

Protein concentration was measured by the method of Bradford [11] with γ-globulin as a standard.

3. Results and discussion

3.1. Chromatographic analysis

In order to determine a great number of analytical samples the HPLC conditions were studied. As shown in Fig. 1, NA and NAA were eluted within 5 min under the conditions described in the Section 2. The retention times for NA and NAA are 4.0 min and 2.5 mm, respectively. As NA and NAA were well separated, the presence of a high NAA concentration (50 μ*M*) as substrate did not interfere with the detection of NA. The amount of NA formed by the enzyme reaction was determined by a calculation of the peak area in comparison with the calibration curve for the standard. The conversion of NAA to NA by deamidase was confirmed from the decrease in the NAA concentration and the appearance of the NA peak. The detector response was linear for NA with concentrations ranging from 10 μ*M* to 10 m*M* (the correlation coefficient; r^2 was 0.999). The detection limit for NA was (signal-to-noise ratio=5) 0.5 μ*M*. Yan et al. [10] described a method for determining the activity of nicotinamide deamidase purified from yeast using HPLC under conditions without the ion-pair regent. Under these HPLC conditions the crude cell extracts from bacteria in our study could not be applied because of the appearance on the chromatogram of peaks which interfered with the NA determination. As shown in Figs. 2 and 3, NA and NAA peaks were completely separated from the interfering substances in the crude cell extract under our HPLC conditions and we were able to determined NA and NAA simultaneously over a wide range.

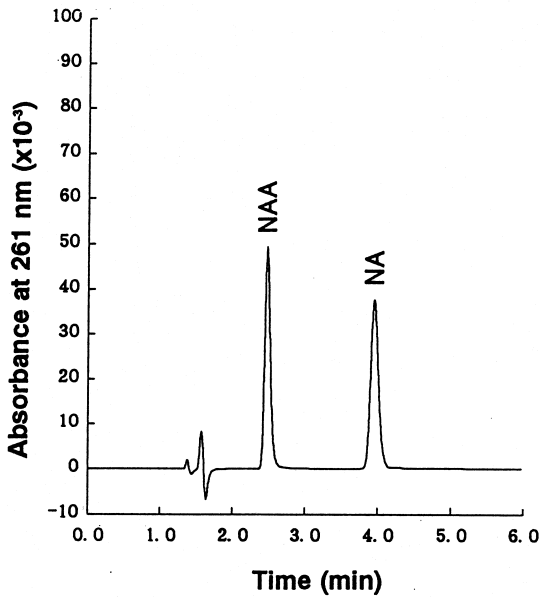

Fig. 1. Typical chromatogram of a standard solution.

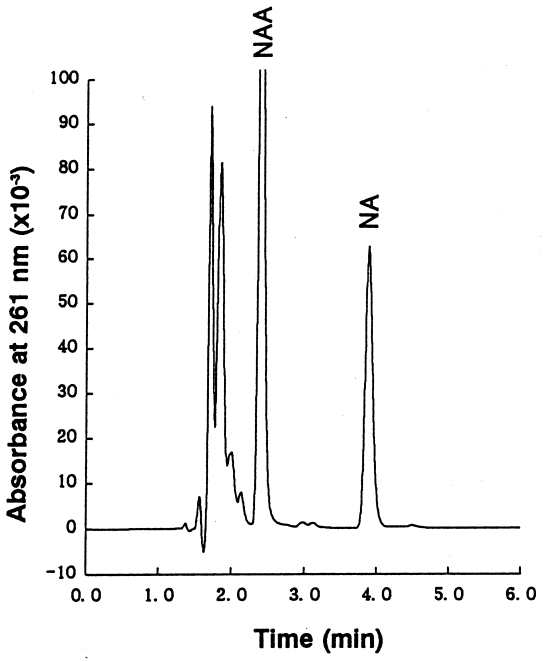

Fig. 2. Chromatogram of the reaction mixture containing crude cell extract from bacteria (*Enterobacter agglomerans*) isolated from meat.

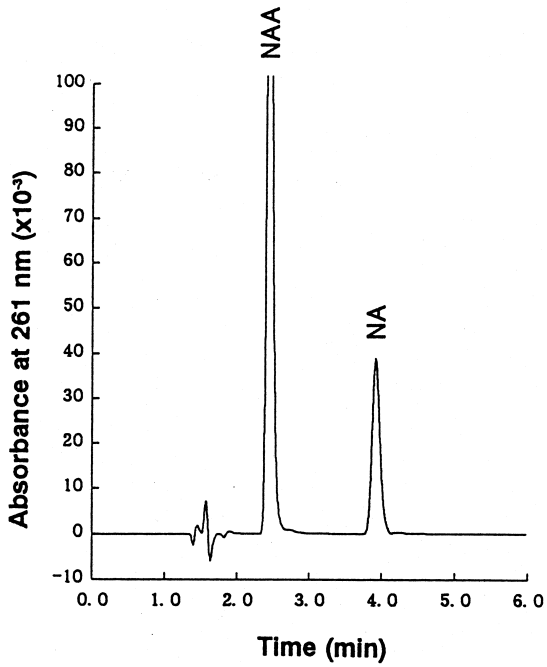

Fig. 3. Chromatogram of the reaction mixture containing partially purified nicotinamide deamidase from bacteria (*Enterobacter agglomerans*) isolated from meat.

3.2. Optimization of conditions for nicotinamide deamidase assay

In order to determine the optimum assay conditions for nicotinamide deamidase activity, an experiment was performed using a partially purified enzyme prepared from *Enterobacter agglomerans*. First, the optimum pH was investigated. The reaction mixture (total volume, 1 ml) containing 100 μl of 10 μ*M* NAA as substrate, 100 μl of enzyme solution (ca. 104 μg), 600 μl of water and 200 μl of 0.5 *M* acetate buffer (pH 4.0–5.5), 0.5 *M* phosphate buffer (pH 6.0–8.0) or 0.5 *M* glycine buffer (pH 9.0–11.0) was incubated at 37°C for 15 min and the deamidase activity estimated from the amount of NA produced. As shown in Fig. 4, the optimum pH was 7.5, i.e. nearly neutral. This optimum pH value was similar to those of the enzyme from other microorganisms (*Aspergillus niger*: pH 7.5 [12], *Mycobacterium avium*: pH 8.3 [9], *Torula crenoris*: pH 7.0 [4], *Escherichia coli*: pH 7.2 [7], *Fleishmann's yeast*: pH 6.5–7.5 [6]). As is evident from the results in Fig. 4, the enzyme reaction was terminated at low pH such as pH 4 or below, which is achieved by the addition of the acetic acid solution after the incubation at 37°C for 15 min. We found that NA produced by the enzyme reaction was stable in the acidic solution at 4°C for 24 h. Therefore a serial analysis of multiple samples is possible using the autosampler after termination of the reaction. The effect of buffer concentration on the enzyme reaction was tested over a range from 0.02 to 2 *M*. No significant difference was found in the NA peak area at any concentration tested (results not shown). The buffer concentration of 0.1 *M* was selected in order to reliably maintain the enzyme solution at pH 7.5.

The time course of NA production during the deamidase hydrolysis reaction is shown in Fig. 5. Under the present assay conditions, the NA production increased with incubation time and the deamidase activity was found to be linear up to 120 min. We did not observe any inhibition of the deamidase by the NA produced, over the range of times tested. In consideration of rapidity and accura-

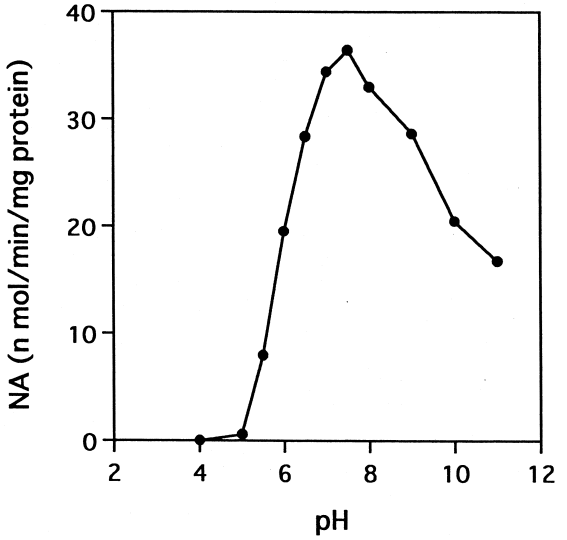

Fig. 4. Effect of pH on nicotinamide deamidase activity. Partially purified enzyme activity of *Enterobacter agglomerans* was measured at 37°C for 15 min by the method described in the text. The buffers used were as follows: 0.1 *M* acetate buffer (pH 4.0–5.5); 0.1 *M* phosphate buffer (pH 6.0–8.0); 0.5 *M* glycine buffer (pH 9.0–11.0). Each data point is the mean ±S.D. of three determinations.

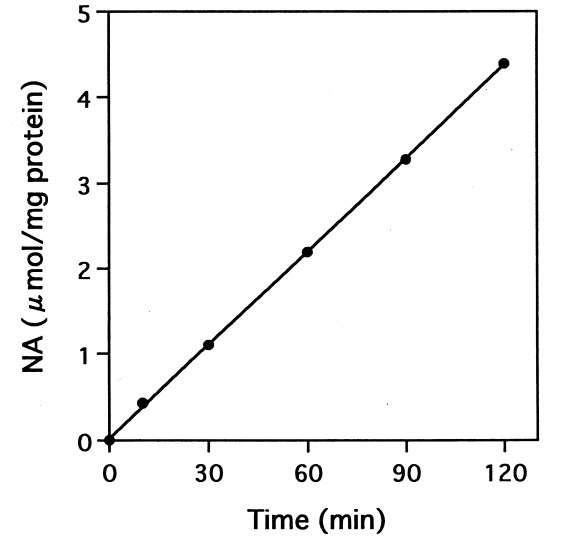

Fig. 5. Time course of NA formation by nicotinamide deamidase. The reaction mixture was prepared as described in the text with 104 μg of the partially purified enzyme from *Enterobacter agglomerans* and 1 m*M* NAA in a total volume of 1 ml, and then incubated at 37°C for various times. Each data point is the mean ±S.D. of three determinations.

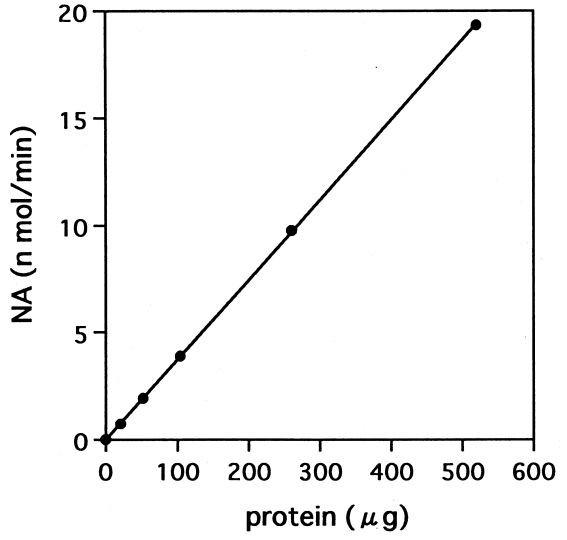

Fig. 6. Linear enzyme activity. Reaction mixtures were prepared as described in the text containing O, 20.8, 52.0, 104.0 260.0 or 520.0 μg of the partially purified enzyme from *Enterobacter agglomerans* and 1 m*M* NAA in a total volume of 1 ml, and then incubated at 37°C for 15 min. Each data point is the mean±S.D. of three determinations.

cy for the assay method, an incubation time of 15 min was chosen.

As shown in Fig. 6, the amount of NA produced by the enzyme reaction increased with the amount of the enzyme in the reaction mixture and this was linear for enzyme concentrations ranging from 20.8 to 520 μg. When replicate determinations of deamidase activity were performed for the reproducibility study, the coefficient of variation was 0.83% (n=6).

3.3. Kinetic studies

Using the partially purified enzyme the kinetic parameters for the enzymatic hydrolysis by nicotinamide deamidase were determined from their Lineweaver-Burk plots. As shown in Fig. 7, the amount of NA production increased with the substrate concentration, and reached a plateau at 1 m*M* or over. Based on these results, we estimated a Michaelis constant (K_m) of 36 μ*M* and maximum rate (V_{max}) value of 37.6 nmol/min/mg for NAA towards nicotinamide deamidase under the present assay conditions. It is known that the K_m values for

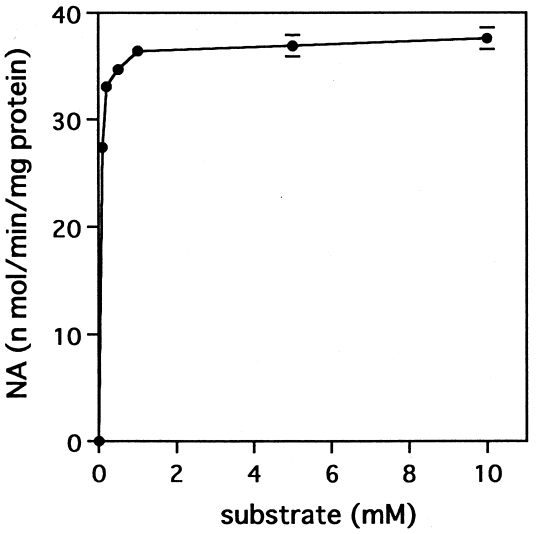

Fig. 7. Effect of the substrate (NAA) concentration on NA formation. Reaction mixtures were prepared as described in the text containing 0, 0.1, 0.2, 0.5, 1.0, 5.O or 10 m*M* and 104 μg of the partially purified enzyme from *Enterobacter agglomerans* in a total volume of 1 ml, and then incubated at 37°C for 3O min. Each data point is the mean±S.D. of three determinations.

microorganisms are lower than those for mammalia. The K_m value for our enzyme was in the same range as those for various microorganisms (5.8–70 μ*M*) [4,6,7,10].

3.4. Application of the method to bacterial strains

Using our assay method we measured the deamidase activity in crude cell extracts prepared from several bacterial strains that in our previous paper had converted NAA to NA [2]. The results shown in Table 1 indicate that these bacterial strains enzymatically converted NAA to NA, and supports the possibility that the appearance of NA in meat during storage was caused by the contaminating bacteria.

Table 1
Nicotinamide deamidase activity of several bacterial strains isolated from meat

Strain	mUnit/mg protein
B-1 (*Enterobacter agglomerans*)	2.0
B-4 (*Enterobacter cloacae*)	1.0
B-10 (*Aeromonas* sp.)	2.1
C4-2 (*Pseudomonas* sp.)	5.0
C4-11 (*Enterobacteriaceae*)	1.4
D5-2 (*Pseudomonas* sp.)	0.9
D5-13 (*Enterobacter amnigenas*)	0.5

In conclusion, since the present method is rapid, simple and reproducible, it can be applied to nicotinamide deamidase from various sources such as microorganisms and mammals. It will be a useful tool for future studies of nicotinamide deamidase activity.

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