Plant Cell Reports (1982) 1: 236–239

Plant Cell Reports
© Springer-Verlag 1982

Separation of Two Distinct S-Adenosylmethionine Dependent N-Methyltransferases Involved in Hordenine Biosynthesis in *Hordeum vulgare*

Eckart Meyer

Lehrstuhl für Biochemie der Pflanzen der Universität Münster, Hindenburgplatz 55, D-4400 Münster, Federal Republic of Germany

Received August 3, 1982

ABSTRACT

Hordenine is biosynthesized in young roots of barley by subsequent N-methylation of tyramine. It was shown that two distinct enzymes are responsible for these methylation reactions. They differed in their pH-optimum, their stability in dependence of the pH-value, and were partially resolved by DEAE-chromatography. More than 200-fold purification and almost complete separation were achieved by elution of the enzymes from an affinity column which was prepared by coupling S-adenosylhomocysteine to amino-hexyl-Sepharose 4 B.

Abbreviations: NMT, N-methyltyramine; SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine; DTE, dithioerythritol

INTRODUCTION

Methyltransferases are involved in the biosynthesis of a great number of secondary plant products, catalyzing O-, C- and N-methylations. In a recent review, Poulton (1981) summarizes the spare results on enzymatic N-methyl transfer reactions in alcaloid biosynthesis. Among the best investigated compounds are phenyl- or indolealkylamines with rather simple but interesting structures due to the fact that they can be methylated twice at their side-chain nitrogen.

Mann et al. (1963 a, 1963 b) have described an enzyme preparation from roots of young barley seedlings which catalyzed the formation of N-methyltyramine (NMT) and hordenine (N,N-dimethyltyramine) by transferring methyl groups from S-adenosylmethionine (SAM) to tyramine. But it was left undecided whether the two successive methylations were performed by one or two enzymes.

Evidence that such reactions might require two distinct enzymes was given by Mack and Slaytor (1978). They partially resolved tryptamine N-methyltransferase and N-methyltryptamine N-methyltransferase activities from Phalaris tuberosa. But a complete separation of two N-methyltransferases acting at the same nitrogen atom has not yet been described in the literature.

Pursuing previous investigations on hordenine metabolism in barley (Meyer and Barz 1978), the present work was aimed to determine the number of N-methyltransferases involved in hordenine biosynthesis and to separate them, if evidence for two enzymes could be obtained.

METHODS

Barley (Hordeum vulgare var. Dura) seeds were soaked in tap water for 24 hours and then grown in trays of moist sand at 25°C. Roots of 4-6 days old seedlings were cut off and ground with 0.1 g PVP/g fresh weight and some quartz sand in 2 parts of 0.05 M Tris/HCl buffer pH 7.5 which contained 50 mM β-mercaptoethanol. Centrifugation at 30,000 x g yielded a clear supernatant (crude extract), which was then fractionated by the addition of solid $(NH_4)_2SO_4$. The pellet of the fraction from 55-70 % $(NH_4)_2SO_4$ saturation was dissolved in buffer and desalted by Sephadex G 25 chromatography. 10 mM Dithioerythritol was added for all further use of this enzyme preparation.

The standard assay contained in a total volume of 0.2 ml 250 mM Tris/HCl pH 8.4, 0.5 mM tyramine (or 2.5 mM N-methyltyramine) and 0.1 mM S-adenosyl-L-(methyl-^{14}C)methionine (18.5 MBq/mmol) and was performed at 27°C. After 30 minutes 0.2 ml 1 M borate buffer pH 10 and 5 ml ethylacetate were added and amines extracted into the organic layer, 4.5ml of which were transferred into scintillation vials and counted with 10 ml of a cocktail containing 8 g PPO in toluene/methanol 4:1(1 l). By this procedure 68 % of the product formed in the assay was recorded. Protein was determined according to Bradford (1976).

SAH was coupled to AH-Sepharose 4 B exactly as described by Sharma and Brown (1978). Enzyme was loaded onto a column with a gel volume of 3.5 ml and eluted at a rate of about 15 ml/hour. Primary washing with 0.05 M Tris/HCl buffer pH 8.4 was followed by elution of protein with 0.5 M NaCl in buffer. N-methyltransferase activities were then eluted with a SAM gradient from 0 to 4 mM in buffer containing 0.5 M NaCl.

0721–7714/82/0001/0236/$ 01.00

RESULTS

Mann et al. (1963 b) had worked with a barley cultivar which contained NMT in much higher amounts than hordenine, and correspondingly these authors found only low levels of NMT methylating enzyme activity. Therefore, a barley cultivar ("Dura") has now been chosen for the enzymatic studies which was found to contain about equal amounts of both amines. Crude extracts from roots of 5 days old "Dura" seedlings indeed methylated both substrates, tyramine and NMT, at almost identical rates. Equally, all other cultivars of barley examined had ratios of N-methyltransferase activities which corresponded to the ratios of their accumulated amines.

Products of the enzyme reactions were characterized by thin layer chromatography in different solvent systems and shown to be exclusively NMT or hordenine depending on the substrate used. With tyramine as substrate, hordenine formation was never observed, because the strong product inhibition by SAH only permitted determination of initial rates. Therefore, amounts of NMT formed were insufficient for subsequent methylation. Furthermore no indication has been obtained for O-methylation of the two substrates, even when crude extracts were used.

Partial purification of the "Dura" enzymes was achieved by ammonium sulphate fractionation in the range of 55-70 % saturation. Yielding about 85 % recovery, the specific activities increased three-fold. This preparation was desalted via a Sephadex G 25 column chromatography and used for most experiments.

Significant loss of activity was observed when enzyme preparations were stored. Especially the NMT methylating activity proved to be very unstable when stored at assay pH. 50 % of this activity were lost within 1.5 hours at room temperature (4.5 hours for tyramine N-methylation).

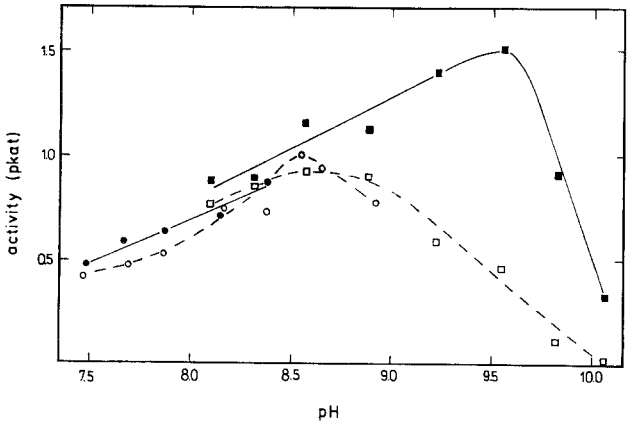

Fig.1. pH-Optima of barley tyramine N-methyltransferase (●—●, ■—■) and N-methyltyramine N-methyltransferase (○--○, □--□) in 250 mM glycine (squares) or glycylglycine (circles) buffer. pH Values were determined in the complete assay mixtures at 27°C.

SH-protecting reagents were absolutely required to conserve some of the enzyme activity . Dithioerythritol (DTE) was found to be twice as effective as reduced glutathione, cysteine or mercaptoethanol respectively (all at 10 mM and 4°C). When DTE was used decay rates were $t_{1/2}$ = 4.5 days for tyramine and $t_{1/2}$ = 1.5 days for NMT methyltransferases. Without protection enzyme activities decreased to 55 and 8 % after 24 h at 4°C. Enzyme activity which had once been lost could not be restored even with DTE concentrations up to 50 mM.

Freezing destroyed both enzyme activities, unless both 10 % glycerol and 10 mM DTE were present. Under these conditions storage at 20°C was possible for weeks and thawing and refreezing hardly affected activities.

Further studies revealed a great influence of the pH value on enzyme stabilities. For practical reasons routine assays were performed at pH 8.4 for both substrates. pH Optima (Figure1) were found to be broad (more than 50 % of maximal activity in a range of almost 2 pH units) but markedly different for tyramine (pH 9.5) and NMT (pH 8.5) methylating activities.

However, buffers with a pH value optimal for enzyme activity proved to be very bad for enzyme stabilities (Figure 2). Enzyme preparations were stored in various buffers with 5 mM DTE at 4°C and assayed at once (100 % value, less than 10 % and not pH dependent difference in activities) and after 4,11,24 and 52 hours under standard conditions (pH 8.4).

For greater clarity only 24 hour data are plotted in Fig. 2. After 11 and 52 hours, plots were almost parallel, namely 20 % above or below the 24 hour data.

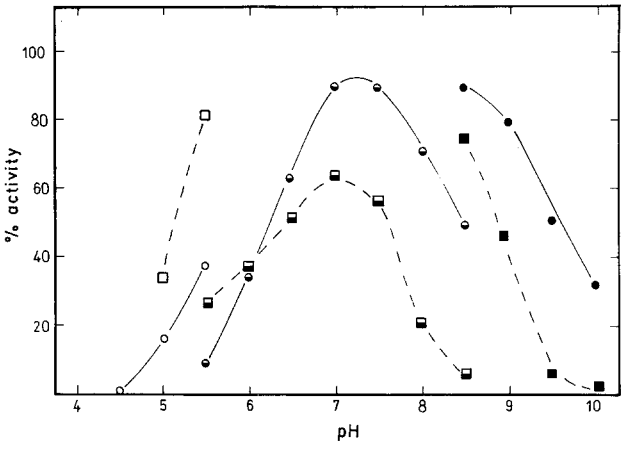

Fig. 2. pH-Dependent stability of barley tyramine N-methyltransferase (circles) and N-methyltyramine N-methyltransferase (squares) in 50 mM citrate (○—○, □--□) Tris/HCl (◒—◒, ◨--◨) and glycine (●—●, ■--■) buffer, all supplemented with 10 mM DTE. Activity was assayed under standard conditions (250 mM Tris/HCl, pH 8.4, 27°C) and plotted as % of initial activity after 24 h storage at 4°C.

In general, NMT methylating activity was lost more rapidly, even between pH 7 and 7.5 which was optimal for stability of both activities. But behaviour at more extreme pH values was different. At basic pH the tyramine methylating activity decreased more slowly while at acidic pH the NMT methylating activity was relatively more stable. As shown in Figure 2, the effects of different buffer ions only influenced the rate of enzyme inactivation but not the general shape of the curves.

The different behaviour of the two obviously distinct N-methyltransferases towards the pH of their environment seemed to increase the chance for their separation by ion exchange chromatography. Therefore, enzyme preparations obtained after ammonium sulphate fractionation and Sephadex G 25 chromatography were loaded onto columns of DEAE-cellulose and eluted under various conditions.

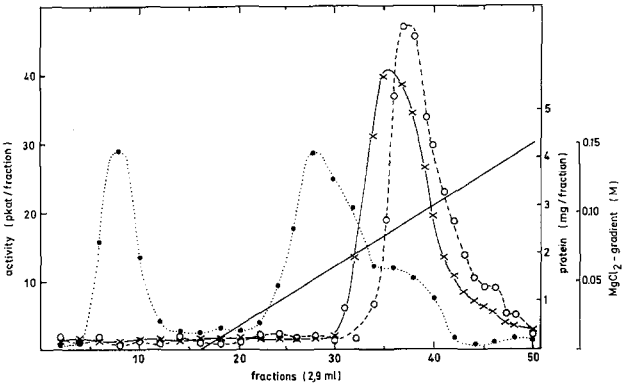

Fig. 3. DEAE-chromatography of barley tyramine N-methyltransferase (X——X) and N-methyltyramine N-methyltransferase (o- -o) as compared to protein elution (●····●) in 20 mM Tris/maleate buffer pH 7 plus a $MgCl_2$ gradient in the same buffer.

The results obtained are represented by Figure 3, where a flat gradient from 0-0.15 M magnesium chloride elutes the NMT methyltransferase later than the tyramine methyltransferase.

The elution pattern of Figure 3 could not be improved by changes of pH, gradient steepness, salt concentration, replacing $MgCl_2$ by NaCl or by increasing buffer molarity. A very similar elution profile was obtained by Mack and Slaytor (1978), when they chromatographed the corresponding tryptamine N-methyltransferases on affinity columns with SAH as the ligand.

The work of Sharma and Brown (1978,1979) not only had given additional indication for the great chances chromatography on SAH-linked Sepharose columns offers for the separation of methyltransferases, but also gives a simple procedure for the preparation of these affinity media. Sharma and Brown first eluted their methyltransferases with buffers of pH 3 (1978) and later with substrate analogues (1979). Since pH 3 irreversibly inactivated the barley methyltransferases, elution with the substrate SAM was tried.

When enzyme preparations were applied onto a column of SAH-sepharose prepared according to Sharma and Brown (1978), some protein but no activity was eluted in the void volume. With buffer alone, nothing eluted off the column, but when 0.5 M NaCl was added, the bulk of the protein was immediately washed off. Enzymatic activity, however, remained on the column, and only eluted with SAM plus NaCl. SAM as sole eluant would not remove any activity.

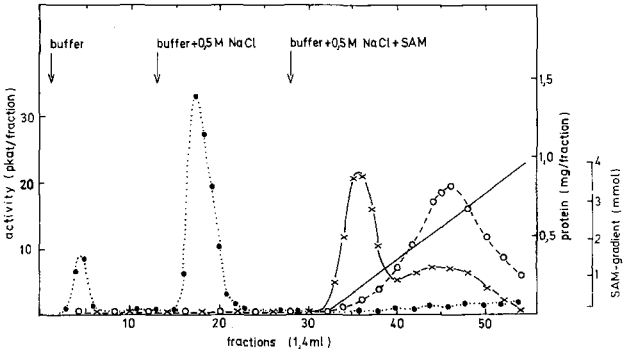

Fig. 4. Chromatography of barley tyramine N-methyltransferase (X——X) and N-methyltyramine N-methyltransferase (o- -o) on SAH-linked AH-Sepharose (3.5 ml gel volume in 0.05 M Tris/HCl buffer pH 8.4). Assays were performed with 100 µl of each fraction and triple amounts of labelled SAM. ●····● indicates protein.

Finally, with the gradient presented in Figure 4, an almost complete separation of tyramine and N-methyltyramine methyltransferases could be achieved. This included an increase in specific activities from 2.4 pkat/mg protein to more than 500 pkat/mg protein. Protein amounts were barely measurable and were probably still overestimated, because SAM slightly interfered with the assay. This can be seen from Figure 4, where the SAM gradient is reflected by the protein data.

DISCUSSION

One of the problems in studies on methyltransferases has been the complete separation of enzymes from the same plant material acting on different substrates. For example, only partial resolution of such enzymes was possible in case of caffeic acid and quercetin methylation in tulip anthers (Sütfeld and Wiermann 1978) and tobacco cell cultures (Tsang and Ibrahim 1979). In spinach chloroplasts these enzymes were shown to be present in different compartments and therefore were separable (Charriere-Ladreix et al. 1981). It is understandable that different methylations at the same molecule create even greater problems. Good results were only recently obtained by Ibrahim and De Luca (1982) who separated three quercetin methylating O-methyltransferases by chromatofocusing. This method had been applied to the barley N-methyltransferases which were shown to have a pI-value < 5. An exact determination had not been possible due to the instability of the enzymes at these pH values. Concerning N-methyltransferases methylation at different positions of xanthin in coffeine biosynthesis has not led to the demonstration of multiple forms of methyltransferase in coffee fruits (Roberts and Waller 1979). These authors stress the lability of the N-methyltransferases and add bovine serum albumin (BSA) for stabilization. But for the barley enzymes BSA had only little effect compared to SH-protecting agents, especially DTE.

The differing pH and stability optima found for the two barley N-methyltransferases are properties which have not yet been described for N-methyltransferases. In addition, stability of the two enzymes was influenced in different ways by acidic or basic pH. While the more basic pH optimum and the greater stability at basic pH agree for the tyramine N-methyltransferase, the slower inactivation of the NMT N-methyltransferase at acidic pH is surprising in view of the still basic pH optimum and the greater overall lability. The work of Mack and Slaytor (1978) was the first to show the existence of two separable N-methyltransferases acting at the same nitrogen. Though they have used SAH affinity chromatography, the resolution obtained for these two enzymes was not much better than that obtained by DEAE-chromatography for the barley N-methyltransferases. As shown in figure 4, it was possible, however, to further improve the SAH affinity chromatography system by using SAM in the elution buffer. The almost complete separation of the two enzymes by this system is further investigated since the necessity of salt in addition to SAM indicates that other effects than pure affinity binding are responsible for the separation.

The tyramine N-methyltransferase activity of the second peak is more likely due to affinity of NMT N-methyltransferase to the substrate tyramine than to another form of N-methyltransferase activity, but this can only be decided when greater quantities of enzymes have been purified and characterized towards their substrate specificities.

The existence of two separable N-methyltransferases in barley roots helps to understand the variance of either preferential hordenine or NMT production in different cultivars of barley. Evidence was obtained that the content of the respective amine is a consequence of the amount of the corresponding N-methyltransferase activity present.

ACKNOWLEDGEMENTS

The skilful technical assistance of Mrs. U. Kopka is gratefully acknowledged.

Further thanks are due to the Deutsche Forschungsgemeinschaft for financial support of this research (Forschergruppe "Sekundäre Naturstoffe/Zellkulturen").

REFERENCES

Bradford MM (1976) Anal Biochem 72:248-254

Charriere-Ladreix Y, Douce R, Joyard J (1981) FEBS Letters 133: 55-58

Ibrahim RK, De Luca V (1982) Naturwissenschaften 69: 41-42

Mack JPG, Slaytor MB (1978) J Chrom 157: 153-159

Mann JD, Mudd SH (1963 a) J Biol Chem 238: 381-385

Mann JD, Steinhart CE, Mudd SH (1963 b) J Biol Chem 238: 676-681

Meyer E, Barz W (1978) Planta med 33: 336-344

Poulton JE (1981) In: Stumpf PK, Conn EE (eds) The Biochemistry of Plants, Vol 7, Academic Press pp 668-723

Roberts MF, Waller GR (1979) Phytochemistry 18: 451-455

Sharma SK, Brown SA (1978) J Chrom 157: 427-431

Sharma SK, Brown SA (1979) Can J Biochem 57: 986-994

Sütfeld R, Wiermann R (1978) Biochem Physiol Pflanzen 172: 111-123

Tsang YF, Ibrahim RK (1979) Phytochemistry 18: 1131-1136