TRITIUM LABELLING OF THAUMATIN I, A SWEET--TASTING PROTEIN FROM *THAUMATOCOCCUS DANIELLII* BENTH, BY REDUCTIVE METHYLATION

H. van der Wel, A. Wiersma and J.N. Brouwer

Unilever Research, Vlaardingen, The Netherlands

Received December 29, 1976
Revised July 1, 1977

SUMMARY

Thaumatin I has been labelled by reductive methylation of four of the ε-amino groups of the lysine residues in the protein with tritiated sodium borohydride.Methylation with unlabelled sodium borohydride showed that up to seven amino groups can be methylated without loss of sweetness intensity.
The yield of the tritium-labelled methylated Thaumatin I was 31% with a tritium incorporation of 4.7%. Its specific radioactivity was 12.8 Ci/mmol.

Keywords: Thaumatin I, sweet-tasting protein, tritium labelling, reductive methylation

INTRODUCTION

Studies of the interaction of Thaumatin I, the sweet-tasting protein from the fruit of *Thaumatococcus daniellii* benth (1), with the gustatory epithelium require radioactive labelling of the protein to a high specific activity.

Recently (2), we achieved a reductive methylation of Thaumatin by the procedure of Means and Feeney (3), without loss of sweetness intensity. De la Llosa et al. (4) and Ascoli and Puett (5) used reductive methylation with tritiated sodium borohydride to obtain a tritiated luteinizing hormone with a high specific radioactivity (26-98 Ci/mmol).

0362-4803/78/0514-0735$01.00

We tritiated the lysine residues of Thaumatin by reductive methylation with tritiated sodium borohydride.

Materials and methods

Thaumatin I was prepared as previously described (1). Tritiated $NaBH_4$ (7.2 Ci/mmol) was purchased from Radiochemical Centre Amersham, England, SP-Sephadex C25 from Pharmacia, Uppsala, Sweden and a molecular sieve 13X from Union Carbide. The other chemicals were of analytical grade from Merck, Germany. The UM-2 ultrafiltration membranes were from Amicon, Oosterhout, The Netherlands.

Amino acid analyses were performed in a Beckman Unichrom Amino acid analyzer using the method of Kuehl and Adelstein (6) for the separation of lysine, monomethyllysine and dimethyllysine. Tritium was determined with a Packard-Tri-Carb liquid scintillation spectrometer, model 3375. Electrophoresis was done on 4x17 cm gelatinated cellulose acetate strips (Cellogel ex Chematron, Milan, Italy) in potassium glycinate (0.03 mol/l; pH 10.5).

Preparation of tritiated methylated Thaumatin I

Thaumatin I (20 mg, 1 µmol) dissolved in 1 ml sodium borate buffer (0.03 mol/l; pH 9.0) was mixed at 0°C with a solution of 0.53 mg tritiated $NaBH_4$ (14 µmol, 85 mCi) in 0.5 ml of the same buffer. In 8 min, 4.2 µl of an aqueous solution of formaldehyde (40%, 55 µmol) was added with vigorous stirring.

This part of the reaction was carried out under a gas flow of a mixture of nitrogen and hydrogen (40/1) in order to transport the excess of tritium gas liberated from the closed apparatus. This gas flow was led through a U-tube filled with 250 mg PtO_2 at 125°C to convert $^3H_2$ into ^3H_2O and subsequently through a U-tube filled with molecular sieve 13X to bind the ^3H_2O.

After standing for 10 min at 0°C, the reaction mixture was diluted with 25 ml water, concentrated on a UM-2 filter to 2 ml, again diluted with 50 ml sodium glycinate buffer (0.02 mol/l; pH 9.2) and concentrated to 2 ml on a UM-2 filter. In this way, most of the unreacted sodium borohydride and exchangeable tritium were removed.

Isolation of tritiated methylated Thaumatin I

The 2 ml solution of the tritiated material was diluted to 10 ml with sodium glycinate buffer (0.02 mol/l; pH 9.2) and chromatographed on a SP-Sephadex C 25 ion-exchanger column using a continuous pH-gradient from pH 9.2 to 10.4 (2). Three fractions were obtained: a front-peak fraction (2 mCi) and two fractions (A and B) consisting of methylated Thaumatins (total activity of these two fractions: 10.5 mCi). Fractions A and B were ultrafiltered on UM 2 Diaflo membranes and subsequently freeze-dried (total activity after ultrafiltration: 7.1 mCi).

As fraction B (7.3 mg; 3.1 mCi) was contaminated with some coloured material and fraction A (6.2 mg; 4.0 mCi) was pure methylated Thaumatin, we used only fraction A for further analysis. The yield of this fraction was 31% with a tritium incorporation of 4.7% and a specific radioactivity of 12.8 Ci/mmol. The freeze-dried tritiated methylated Thaumatins were stored at -20°C. The stability of the methylated Thaumatin from fraction A was determined by two different methods. Firstly, by gel electrophoresis on Cellogel, a rapid method requiring only small quantities of material (15 µg). Secondly, by small-scale ion-exchange chromatography on SP-Sephadex C25 (Bed volume 0.3 ml). After application of the sample, the column was washed with a sodium glycinate buffer (0.02 mol/l; pH 9.2) until no radioactivity was measured. The pure methylated Thaumatin was subsequently eluted with a sodium glycinate buffer (0.02 mol/l; pH 10.4). After a period of five months, 80% of the methylated Thaumatin was still intact.

Distribution of the radioactivity in the lysine residues of fraction A of the tritiated methylated Thaumatin I

Fraction A was hydrolysed (HCl, 5 mol/l) in a sealed tube under nitrogen at 110°C for 22 h and evaporated to dryness. All radioactivity was recovered indicating that the preparation was free from labile tritium.

The distribution of the radioactivity was determined, as well as the amino acid residues per mol in the fractions obtained from the amino acid analysis according to Kuehl and Adelstein (Table 1). It appears that 92% of the tritium label is present in mono- and dimethyllysine residues of the molecule.

Table 1. Analysis of the hydrolysate of fraction A of tritiated methylated Thaumatin I. Distribution of the radioactivity and the amino acids over the fractions from the amino acid analyzer column

fraction number	amino acid	residues/mol	radioactivity/%
1 - 12	-	-	0.06
13 - 21	acidic and neutral	155	3.4
22 - 38	tyrosine/phenyl alanine	17	0.11
39 - 76	-	-	0.36
77 - 81	lysine	6	0.03
82 - 85	-	-	0.14
86 - 89	monomethyllysine	1	12.8
90 - 96	dimethyllysine	3	79.2
97 - 124	-	-	1.9
125 - 131	ammonia	-	0.11
132 -	arginine	11	1.6

DISCUSSION

According to Rice and Means (7), reductive methylation is achieved by the addition of two hydrogen atoms to the double bond of a Schiff base:

$$P\text{-}NH_2 + 2\ HCHO + \tfrac{1}{2}\ NaBH_4 \longrightarrow P\text{-}N(CH_3)_2 + \tfrac{1}{2}\ Na^+ + \tfrac{1}{2}\ H_2BO_3^- + \tfrac{1}{2}\ H_2O$$

One $-N(CH_3)_2$ group needs $\frac{1}{2}$ mol $NaBH_4$.

From the radioactivity percentages in Table 1, we calculated the presence of 2.8 dimethyllysine residues and 0.9 monomethyllysine residues. This agrees very well with the figures obtained from the amino acid analysis. So, four of the ten ε-NH_2 groups in the molecule (8) are methylated. The molar ratio of protein to formaldehyde was here 1 to 55.

In the methylation of Thaumatin I with unlabelled $NaBH_4$ (2), the molar ratio of protein to formaldehyde was 1 to 86 which resulted in the methylation of seven free NH_2-groups without loss of sweetness. Therefore, it is certain that the tritiated methylated Thaumatin I has the same sweetness intensity as the untreated Thaumatin and that it is suitable for biological and autoradiographic experiments.

ACKNOWLEDGEMENT

Thanks are due to Dr. I. Heertje and Messrs. H. van Tilborg and K.P. Hissink for their technical advice in radioactive work and to Mr G. Richters for carrying out the amino acid analysis.

REFERENCES

1. Van der Wel, H. and Loeve, K. - Eur. J. Biochem. 31: 221 (1972).

2. Van der Wel, H. and Bel, W.J. - Chem. Senses Flavor, 2: 211 (1976).

3. Means, G.E. and Feeney, R.E. - Biochemistry 7: 2192 (1968).

4. De la Llosa, P., Marche, P., Morgat, J.L. and De la Llosa-Hermier, M.P. - FEBS Lett. 45: 162 (1974).

5. Ascoli, M. and Puett, D. - Biochim. Biophys. Acta 371: 203 (1974).

6. Kuehl, W.M. and Adelstein, R.S. - Biochem. Biophys. Res. Commun. 37: 59 (1969).

7. Rice, R.H. and Means, G.E. - J. Biol. Chem. 246: 831 (1971).

8. Van der Wel, H. (1974) - Symposium: Sweeteners (Inglett, G.E. ed.) pp. 194-203. AVI, Westport, Connecticut, 1974.