PURIFICATION OF HIGHLY ACTIVE MILK XANTHINE OXIDASE BY AFFINITY CHROMATOGRAPHY ON SEPHAROSE 4B/FOLATE GEL

Takeshi NISHINO, Tomoko NISHINO and Keizo TSUSHIMA

Department of Biochemistry, Yokohama City University School of Medicine, Urafune-cho, Minami-ku, Yokohama 232, Japan

Received 9 July 1981

1. Introduction

It is well known that an inactive form of xanthine oxidase, the desulfo-form, is present in milk xanthine oxidase preparations, in addition to an inactive form lacking molybdenum [1–4]. The existence of the desulfo-form in the enzyme preparations is considered to be caused by spontaneous release of sulfur during storage or purification. This inactive form is also known to be caused by treatment of the enzyme with cyanide [5].

To understand the proper mechanism of action of this enzyme, it is important to obtain an enzyme preparation essentially free of inactive forms.

An affinity chromatography method for resolution of the active and inactive forms of xanthine oxidase was reported in [6]. An allopurinol analogue was used as an affinity ligand [6]. In [6], only the active enzyme, in a reduced form, was bound to the column and could be eluted after reoxidation. Despite the extreme value of affinity chromatography, preparation of the enzyme by their method is not easy, because it involves difficult chemical syntheses and chromatography under anaerobic conditions.

Here, we describe a much easier method for purification of highly active enzyme by affinity chromatography, in which resolution of the sulfo- and desulfo-enzymes is achieved. We adopted folate [7], a commercially available competitive inhibitor, as a ligand instead of the allopurinol analogue.

2. Materials and methods

Xanthine oxidase was prepared from cow's milk by the method in [8] with minor modifications. Cysteine (5 mM), salicylate (1 mM) and EDTA (0.2 mM) were added to the extraction buffer of Na_2HPO_4 (0.2 M). Final ammonium sulfate fractionation was performed between 30–45% saturation instead of 33–42% saturation as in the original method.

Abbreviations: DMF, dimethylformamide; EDC, *N*-ethyl-*N'*-3 dimethylaminopropyl carbodiimide; AFR, activity flavin ratio

2.1. *Preparation of Sepharose 4B/folate gel*

AH-Sepharose (10 g) obtained from Pharmacia, was swollen in 0.5 M NaCl and washed with 1 liter of 0.5 M NaCl. Washed AH-Sepharose was mixed with 60 ml 2 mM folate in 50% DMF, the pH of which had been pre-adjusted to 5.8 with dilute NaOH. Then 400 mg EDC, obtained from Fluka, was added and the mixture was gently stirred overnight at room temperature in the dark. The gel was washed sequentially with 200 ml of 50% DMF at pH 7.0, 500 ml 0.01 M NaOH, 500 ml 0.1 M Tris–HCl at pH 7.0, and finally with 1 liter of distilled water. The Sepharose 4B/folate gel was stored at 4°C in the dark.

Oxipurinol was synthesized as in [9]. Allopurinol was obtained from Sigma.

Enzyme activity was measured spectrophotometrically at 295 nm and at 25°C [4] using a Shimazu 140 spectrophotometer. Catalytic activity was expressed as $AFR^{25°C}$ [10].

The following buffer mixtures were used for affinity chromatography:

(A) Mixture of 20% 0.1 M pyrophosphate buffer (pH 8.5) containing 0.2 mM EDTA and 80% 0.05 M Tris–HCl buffer (pH 7.8) containing 0.2 mM EDTA.

(B) Mixture of 30% of 0.1 M pyrophosphate buffer (pH 8.5) containing 0.2 mM EDTA and 70% 0.05 M Tris–HCl buffer (pH 7.8) containing 0.2 mM EDTA.

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50

3. Results

3.1. *Purification of xanthine oxidase*

All procedures were performed at 4°C unless otherwise indicated. Milk xanthine oxidase (13 ml) with an absorbance at 450 nm of 2.1 (obtained by the modified method of Ball) was dialysed against buffer mixture (A) then applied to the Sepharose 4B/folate column (2 × 6 cm) pre-equilibrated with buffer mixture (A). Almost all the material having absorbance at 450 nm was adsorbed on this column. After washing off the impurities with buffer mixture (B) the enzyme was eluted with buffer mixture (B) containing 0.5 mM hypoxanthine as shown in fig.1. The fractions containing enzyme activity were pooled soon after elution and solid ammonium sulfate was added to 60% saturation. Precipitated enzyme was collected by centrifugation and dissolved in a minimal volume of 0.1 M pyrophosphate (pH 8.5) containing 0.2 mM EDTA. The enzyme was dialysed against 250 ml 0.1 M pyrophosphate buffer (pH 8.5) containing 0.2 mM EDTA. The dialysis buffer was changed once. As shown in table 1, the absorbance ratio (A_{280}/A_{450}) of the dialysed sample was 5.2, suggesting that the enzyme was almost pure [4], but the *AFR* was still 126, suggesting that the inactive form was not removed by this affinity chromatography step.

3.2. *Resolution of active and inactive enzyme by affinity chromatography*

Since only the active sulfo-enzyme (reduced form) can bind pyrazolo [3,4-d]pyrimidine derivatives [3], we chose to complex the sulfo-enzyme with allopurinol or oxipurinol in order to block the active site and prevent binding to the folate ligand of the affinity column. Enzyme (2 ml) (A_{450} = 6.0, obtained from the first affinity column) in 0.1 M pyrophosphate buffer (pH 8.5) containing 0.2 mM EDTA was placed in the body of a Thunberg tube. The side-arm contained 20 μl of 10 mM oxipurinol and 50 μl 10 mM xanthine, both dissolved in 0.01 M NaOH. The system was made anaerobic, the reagents were mixed with the enzyme anaerobically and incubated at room temperature for 1 h. Under aerobic conditions, the oxipurinol-treated enzyme was quickly passed through Sephadex G-25 which was equilibrated with buffer mixture (A) to remove xanthine and excess oxipurinol. The oxipurinol-treated enzyme was then applied to the Sepharose 4B/folate affinity column (2 × 6 cm) equilibrated with buffer mixture (A). As shown in fig.2, only the oxipurinol-inhibited sulfo enzyme was eluted by buffer mixture (B). On the other hand desulfo-enzyme (low activity) remained on the column and was eluted with buffer mixture (B) containing hypoxanthine. Highly active enzyme fractions (*AFR* 205–190) were pooled, and 1 mM salicylate and 50 μM ferricyanide (final concentrations) were

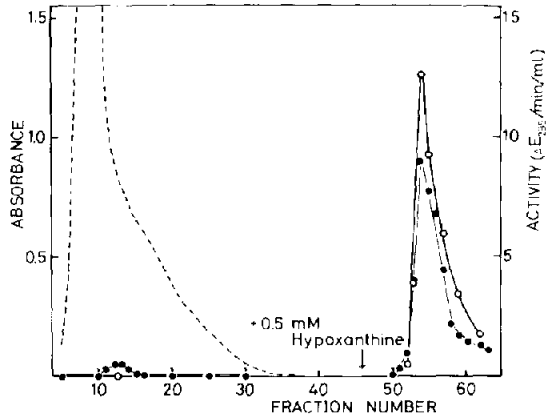

Fig.1. Purification of milk xanthine oxidase by affinity chromatography on Sepharose 4B/folate. After washing off the impurities with buffer mixture (B) the enzyme was eluted with buffer mixture (B) containing 0.5 mM hypoxanthine. Fractions of 5 ml were collected. (– – –) absorbance at 280 nm; (•) absorbance at 450 nm; (○) enzyme activity expressed as change in absorbance at 295 nm . min^{-1}. ml^{-1}.

Table 1
Purification and resolution of active and inactive xanthine oxidase

	A_{280}/A_{450}	$AFR^{25°C}$
Before 1st affinity chromatography	11.0	130
After 1st affinity chromatography	5.2	126
After 2nd affinity chromatography		
Active fractions pooled	5.0	195
Inactive fractions pooled	5.1	38.1

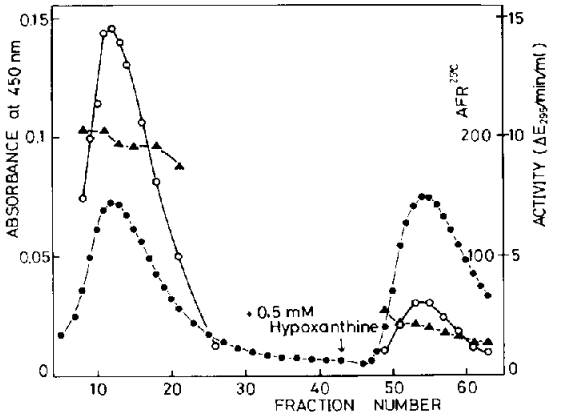

Fig.2. Resolution of active and inactive xanthine oxidase by second affinity chromatography on Sepharose 4B/folate. The oxipurinol-treated enzyme was rechromatographed on the same affinity column as described. The activity (○) was measured after treatment with ferricyanide; (●) absorbance at 450 nm; (▲) *AFR* value at 25°C. Fractions of 5 ml were collected.

added to the solution [4]. The mixture was stirred gently at room temperature in the dark in order to reactivate the oxipurinol-inhibited enzyme. After complete reactivation, when catalytic activity no longer increased, ammonium sulfate was added to 60% saturation and the resultant precipitate was collected by centrifugation. The precipitated enzyme was dissolved in a minimal volume of 0.1 M pyrophosphate (pH 8.5) containing 0.2 mM EDTA and was passed through a Sephadex G-25 column equilibrated with the same buffer. The $AFR^{25°C}$ value of the pooled enzyme was 195, as shown in table 1, a value which is very close to the maximum values reported in [2,4,6]. On the other hand, the $AFR^{25°C}$ value of the pooled inactive fractions was 38.1. This residual activity could be due to some amount of active monomer in the dimeric molecule.

4. Discussion

As folate is known to be a competitive inhibitor of xanthine oxidase [7], an AH-Sepharose 4B gel to which folate was bound, was used for affinity chromatography. Both the sulfo- and desulfo-forms of the enzyme bound to the column and eluted with hypoxanthine. However, after treatment of the enzyme with oxipurinol and xanthine (these compounds could be replaced by allopurinol alone), the sulfo-enzyme passed through the column, while desulfo-enzyme remained on the column. The desulfo-enzyme could then be eluted with hypoxanthine. It should be noted that desulfo-enzyme could bind hypoxanthine and folate, suggesting that the sulfur atom may not be involved in the formation of enzyme–substrate complex. This confirms reports with [^{14}C]hypoxanthine [11] and with mercaptopyrazolo-pyrimidine derivatives [6].

Existence of non-functional enzyme in the enzyme preparation was first suggested by Morell [1]. Two forms of non-functional enzyme were subsequently indicated: one form containing a full complement of the oxidation–reduction groups; and another form lacking molybdenum [2,4,5]. However, final proof of the correctness of Morell's hypothesis came with the resolution of the active and inactive forms of the enzyme by affinity chromatography [6]. An analogue of allopurinol was used as an affinity ligand [6]. We confirm the conclusion in [6] that xanthine oxidase preparations contain non-functional enzyme which can be resolved into high and low activity fractions. The enzyme which we obtained had an $AFR^{25°C}$ value between 190 and 205. These values are very close to the extrapolated 100% active value (AFR = 210) in [3], as well as being nearly identical to the maximum value (AFR = 205) obtained [6]. The extremely high specific activity of our preparation suggests that both the desulfo- and demolybdo-forms of inactive enzyme have been essentially eliminated.

Our affinity column chromatography on Sepharose 4B/folate has great advantages over that in [6]; that is, affinity gel can be prepared easily by using commercially available compounds, anaerobic conditions are not necessary for performing chromatography and the column is applicable to an early stage in the enzyme purification.

Acknowledgements

We would like to express our gratitude to Drs V. Massey, O. Lockridge and L. Schopfer, University of Michigan, for helpful discussions. This research was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science and Culture.

References

[1] Morell, D. B. (1952) Biochem. J. 51, 657–666.

[2] Hart, L. I., McGartoll, M. A., Chapman, H. R. and Bray, R. C. (1970) Biochem. J. 116, 851–864.
[3] Massey, V., Komai, H., Palmer, G. and Elion, G. B. (1970) J. Biol. Chem. 245, 2837–2844.
[4] Massey, V., Brumby, P. E., Komai, H. and Palmer, G. (1969) J. Biol. Chem. 244, 1682–1691.
[5] Massey, V. and Edmondson, D. (1970) J. Biol. Chem. 245, 6595–6598.
[6] Edmondson, D., Massey, V., Palmer, G., Beacham, L. M. iii and Elion, G. B. (1972) J. Biol. Chem. 247, 1597–1604.
[7] Kalckar, H. M. and Klenow, H. (1948) J. Biol. Chem. 172, 349–350.
[8] Ball, E. G. (1939) J. Biol. Chem. 128, 51–67.
[9] Robins, R. K. (1956) J. Am. Chem. Soc. 78, 784–790.
[10] Avis, P. G., Bergel, F. and Bray, R. C. (1955) J. Chem. Soc. 1100–1105.
[11] Fridovich, I. and Handler, P. (1958) J. Biol. Chem. 231, 899–911.