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Note

Application of industrial autofocusing in the isolation of uricase

T. DOBRÁNSKY* and O. SOVA
Institute of Animal Physiology of the Slovak Academy of Sciences, Dukelských hrdinov 1/B, 040 01 Košice (Czechoslovakia)
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In recent years, much attention has been paid to acquiring flavin-enzymes, mainly for their peroxide producing ability, which is very important for industry. Uricase (EC 1.7.3.3), belonging to the above-mentioned group of enzymes, is of great importance for the uric acid level estimation in biological materials in clinical and biochemical laboratories.

Earlier works indicated that uricase was probably a ubiquitous enzyme in the animal kingdom, but further studies showed that uricase was present also in several microbes[1–17]. It is more important to find a suitable technique for uricase isolation than to search for the best producer.

The repertoire of techniques used in uricase isolation includes mainly various sytems of salt precipitation, ion-exchange column chromatography, gel filtration, ultracentrifugation, extraction, etc.[15–26].

The present paper deals with the possibility of isolating uricase by the application of a novel method, industrial autofocusing[29,30], which has been used with success in the isolation and purification of other materials[31]. The principles of this method have been reported elsewhere[29].

MATERIALS AND METHODS

Ten microbial sources of uricase were tested as follows: in 3 g of culture sediment, the protein content and uricase activity[28] were estimated. According to the results obtained, *Candida utilis* was finally selected as the model, as indicated in Table I.

Yeast cells are grown at 37°C by rigorous aeration for 24 h in 20 l of solution containing 5 g of yeast extract, 20 g of glucose, 1 g of amonium sulphate and 1 g of uric acid per litre of distilled water. After cultivation, the cells are harvested by centrifugation and disintegrated by a Tesla sonifier at 22 kHz four times per minute at 4°C. The solution is then clarified from the cell debris by repeated centrifugation at 4°C and 10000 *g* for 20 min and the conductivity of the supernatant is determined under 800 μS/cm by the addition of cold distilled water. Because the enzyme is often unstable by virtue of protein degradation during the long-lasting purification procedure, immediately after adjustment of the conductivity the solution is stabilized with half the volume of a 10% solution of oxidated dextran[27]. The final mixture is

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TABLE I

SELECTION OF MICROBIAL SOURCES FOR URICASE ISOLATION

Strain	*Czech. coll. number*	*Total protein (mg/3 g)*	*Total activity (nkat)*	*Specific activity (nkat)*
Alternaria sp.	3060	24.50	16.0	0.65
Penicillium sp.	3057	32.50	00.0	0.00
Aspergillus niger	3051	29.50	32.0	1.08
Aspergillus glaucus	3056	17.50	00.0	0.00
Bacillus cereus	2048	93.00	00.0	0.00
Bacillus subtilis	2034	11.50	4.0	0.35
Bacillus megatherium	2033	57.00	4.0	0.07
Enterobacter aerogenes	2028	4.50	00.0	0.00
Candida lipolytica	1120	54.00	13.0	0.24
Candida utilis	1107	36.50	38.0	1.04

poured into an autofocuser[31] of 1 l in volume. The autofocusing is carried out at 4°C at 3 W with an electric field strength varying from 200 to 1000 V until the current decreases to zero, for about 36 h. Then, the power is switched off and the vessel opened. The autofocused solution is divided into twenty equal fractions and the pH, protein concentration and uricase activity are determined[28]. The fractions containing uricase activity are pooled and loaded onto a 45 × 2 cm I.D. Sephadex G-100 column equilibrated in 0.01 *M* borate buffer (pH 8.5). The column is washed at 4°C by a flow of 100 ml/h and 5-ml fractions are collected by an automatic fraction collector. All fractions are tested for protein content and uricase activity as above[28], and the active fractions are pooled for the final study of yield.

RESULTS

Fig. 1 shows the autofocusing results from the separation of stabilized supernatant obtained after sonication. During autofocusing, the natural pH gradient is automatically attained by the focused solution. The proteins are focused into several, well-characterized peaks. The bulk volume of proteins is focused to pH 3.15. Fractions containing uricase activity occurred at pH 10.34 and 11.15.

As indicated in Fig. 2, after Sephadex gel filtration, about 50% of the total protein obtained by autofocusing is found in the void volume, while the uricase is retarded in fractions 41–43.

Finally, in Table II, the protein balance and uricase yield at every step used in the purification process are summarized and compared with those of other methods[11,13,15,17].

DISCUSSION

The automatic creation of the natural pH gradient by the supernatant from the sonicated cells is usual in autofocusing. Irrespective of the undulation or steps in

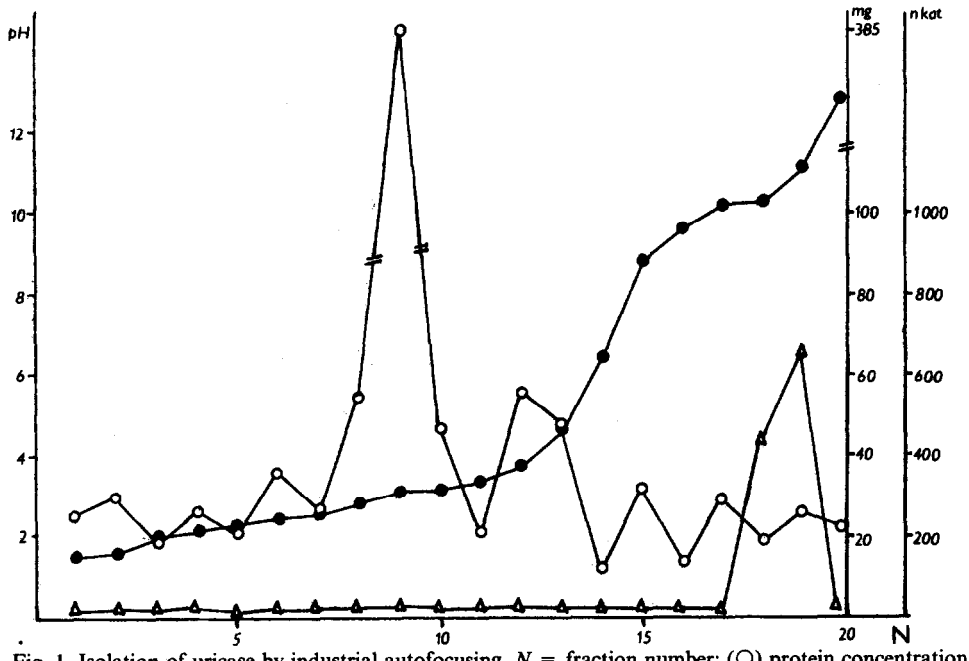

Fig. 1. Isolation of uricase by industrial autofocusing. N = fraction number; (○) protein concentration in mg/fraction; (●) pH gradient; (△) activity of uricase in nkat.

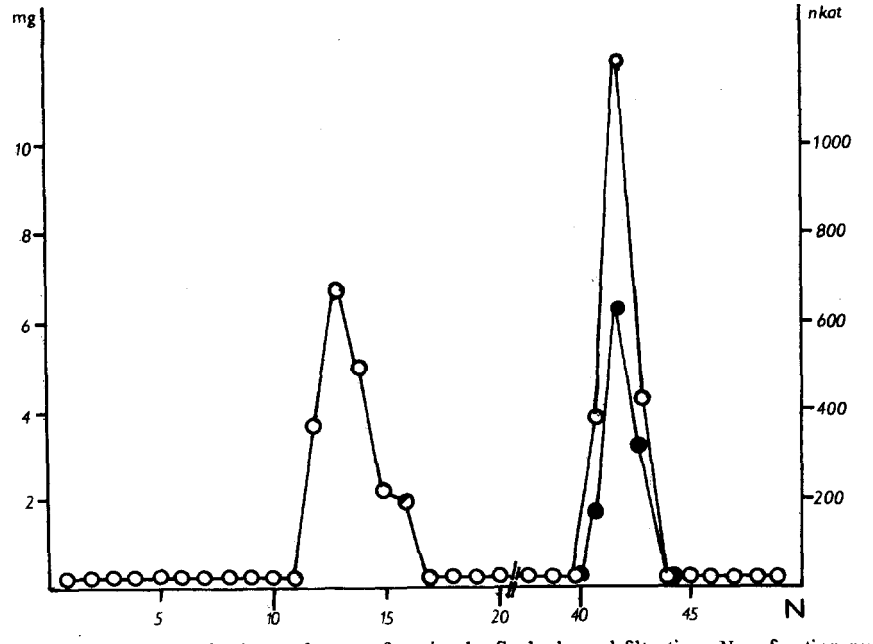

Fig. 2. Purification of uricase after autofocusing by Sephadex gel filtration. N = fraction number; (○) protein concentration in mg/fraction; (●) activity of uricase in nkat.

TABLE II

PURIFICATION OF URICASE BY AUTOFOCUSSING (A) IN COMPARISON WITH THAT OF ANOTHER METHOD (B)[17]

Step	*Total protein (mg)*	*Uricase total activity (nkat)*	*Specific activity (nkat)*	*Purification*	*Recovery (%)*
(A)					
Sonication	–	–	–	–	–
Centrifugation	950.0	1220.00	1.28	1	100
Autofocusing	45.6	1110.00	24.34	19	91
Sephadex pool	20.0	1100.00	55.00	43	90
(B)					
Sonication	–	–	–	–	–
Centrifugation	394.0	494.20	1.26	1	100
$(NH_4)_2SO_4$ precipitation	295.0	480.00	1.62	1.29	97
Ultrafiltration	240.2	471.50	1.96	1.55	95
Ion-exchange chromatography	88.2	450.40	5.10	4	91
Ultrafiltration	70.4	441.00	5.77	4.8	89
Sephadex pool	9.5	415.00	44.00	35	84

the pH gradient, the proteins reach an appropriate p*I* along the pH gradient. The peaks are sufficiently far apart from each other and the void volume is far enough from the peak to show the substantial part of the uricase activity. This step in the isolation is liable to use a large amount of starting material (as many as 3 g of protein per 100 ml). For uricase autofocusing it is very suitable because the p*I* of uricase differs from that of the bulk of the proteins. It must be emphasized that this method should not be used in such cases where the highest activity of the desirable enzymes corresponds with the main peak of proteins.

The last step, gel filtration, in combination with autofocusing is very suitable, because these two methods separate the proteins according to two different criteria, molecular weight and isoelectric point. Table II indicates the use of autofocusing in uricase isolation in comparison with that of current methods using salt precipitation, ion-exchange chromatography and gel filtration. While the modified two-step isolation including autofocusing and gel filtration purifies the uricase 43-fold with 10% loss of total activity, during the usual three-step isolation the uricase is purified only 35-fold with 16% loss of activity. Further advantages of autofocusing are its simplicity, higher yield and production, and its cheapness, as only distilled water and no other chemicals are used for the separation. In this experiment, we used a 20-l culture and an autofocuser of 1 l volume (Realizing Centre, Slovak Academy of Sciences, Košice, Czechoslovakia), but it is also possible to start with a larger amount of material using an autofocuser of 10 l capacity or even larger.

The purity of the product obtained is in good agreement with that of previous reported experiments[11,13,15,17], as shown by gel filtration which is the last step in all systems of uricase isolation.

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