Microbiological Determination of the Amino Acids of Xanthine Oxidase (34725)

NORMA REPETTO DE AMIT AND ELIDA CASTRO DE DUGROS
(Introduced by J. R. Totter)

Department of Biochemistry Faculty of Chemistry, and Department of Biochemistry Faculty of Medicine, Montevideo University, Montevideo, Uruguay

Xanthine oxidase is an enzyme involved in the last steps of the catabolism of the nucleic acids, catalyzing the oxidation of hypoxanthine and xanthine to uric acid. It has been studied extensively by Ball (1) and obtained in crystalline form by Avis *et al.* (2). It is a metal-flavoprotein, the prosthetic nucleus of which is partly formed by FAD, Fe, and Mo. In addition, the spectral analysis indicates another chromophoric group, not yet identified.

Determination of flavin in the enzyme was accomplished by Ball (3) and also by Corran and co-workers (4). The presence of Mo was established independently by Green and Beinert (5) and by Totter *et al.* (6); Richert and Westerfeld (7) showed the presence of bound Fe.

The relationship of Fe to the flavin in xanthine oxidase has been studied by Fridovich and Handler (8). These authors schematize the active site of the enzyme as being formed by two molecules of FAD with different properties. Two types of FAD had already been established by Mackler and coworkers (9) and by Morell (10).

Xanthine oxidase obtained from mammalian liver and from milk are similar but not identical, presenting differences in catalytic activity and in the relative amounts of Fe and Mo. There is no satisfactory explanation at present of these differences and we are studying the protein part of the enzymes from different natural sources: chicken liver, beef liver, and milk.

Results of the determination of the amino acids of highly purified preparations of the enzyme obtained from fresh, non-pasteurized buttermilk are presented here. The determinations were carried out by microbiological and by ion-exchange (Beckman/Spinco Model 120) methods and a comparison was made indicating that the microbiological methods are sufficiently accurate in many cases to complete successfully with the ion-exchange method.

Materials and Methods. Milk xanthine oxidase was prepared following the procedure of Horecker and Heppel (11). The activity was assayed by the spectrophotometric method of Kalckar (12), using xanthine as substrate in a pyrophosphate buffer of pH 8.5, and measuring the uric acid formation at 295 mμ (13).

Protein in the purified enzyme was determined by spectrophotometry (14), employing the biuret reaction (15) and from Kjeldahl nitrogen values; the enzyme content of nitrogen was assumed to be 16.3% (16).

Constancy of the specific activity, the protein–flavin relation found by spectrophotometry, determination of the isoelectric point and electrophoretic homogeneity at different pH values were considered as criteria of purity of the enzyme. Electrophoretic homogeneity was determined by zone electrophoresis, employing 2 mA and 120 V for 12 hr and a medium of Whatman filter paper No. 1 in either 0.1 *M* phosphate buffer (pH 5.7–8.0) or 0.1 *M* acetate buffer (pH 4.0–5.6). Samples of purities between 96 and 98% were used for the analyses. Characteristic values of the absorption spectra and enzymatic activity are shown in Table I.

With the object of obtaining the whole of the amino acids, these preparations were submitted to three types of hydrolysis: acid, alkaline, and enzymatic. As a control for the methods of determination, hydrolysis of pure

TABLE I. Characteristics of Xanthine Oxidase Samples.

$\frac{A_{280}}{A_{450}}$[a]	Activity[b]	Sp act[c]	Protein (mg/ml)
5.0	117	12.2	9.6
5.1	90	10.3	9.0
5.0	95	9.2	10.2
5.0	112	12.5	9.0

[a] A_{280}/A_{450} = protein/flavin relation.

[b] Activity = rate of increase in absorbance at 295 nm/min. It represents the formation of uric acid per minute in the presence of xanthine.

[c] Specific activity = (activity/min)/(mg of protein) = activity per milligram of protein.

casein was carried out simultaneously in a similar manner.

Acid hydrolysis. Protein was hydrolyzed in a flask with adequate stopper by autoclaving at 15 lb pressure for 10 hr with 2.5 *N* HCl (100 mg of protein with 2.5 ml of acid). The product was adjusted to pH 6.8 by adding 2.5 *M* sodium acetate solution and sodium hydroxide solution.

Alkaline hydrolysis. Hydrolysis was accomplished by autoclaving the protein with 5 *N* NaOH for 8 hr at 15 lb pressure (100 mg of protein with 5 ml of NaOH). The hydrolysate was then adjusted to pH 6.8 by addition of hydrochloric acid and sodium acetate solution.

Enzymatic hydrolysis. The preparation for enzymatic digestion was acidified with 0.10 *N* H_2SO_4 and submitted to peptic digestion at 37° for 8 hr (100 mg of protein with 1 mg of pepsin 1/10,000). The pH was then adjusted to 8.4, followed by another digestion with trypsin 1/300 for 8 hr at 40°. After adjustment to pH 7.8, digestion was continued with erepsin at 40° for 48 hr (100 mg of protein with 10 mg of erepsin). In a similar fashion to the products of the other types of hydrolysis, this was brought to pH 6.8 and to volume. N. B. C. Research Biochemicals (pepsin, Trypsin, and erepsin) were used.

Determination of the amino acids in the various hydrolysates was carried out by chromatographic, ion-exchange and microbiological methods.

Chromatographic method. A chromatographic study of the various products of hydrolysis was made on mono- and bidimensional paper, using butanol–acetic acid–water (4–1–1) as solvent for the first run and phenol (80% solution) for the second one. Recognition of the amino acid spots was made by treatment with Ninhydrin (17). This method was developed with qualitative purposes.

Ion-exchange method. The Spinco method was followed, using Beckman/Spinco Model 120 B. (18).

Microbiological method. In order to apply this method, it was necessary to study different lactic acid producing microorganisms, such as *Streptococcus fecalis, Leuconostoc mesenteroides,* and *Lactobacillus arabinosus.* Since there are strains more or less sensitive to certain amino acids in a single species of microorganism, the sensitivities of different bacteria were first determined. Suitable bacterial strains as well as the most convenient medium for each amino acid were selected. For this purpose, a division into four groups was made (Table II).

Assays were accomplished after incubation at 37°, followed either 24 hr later by turbidimetry (using Coleman photocolorimeter with filter No. 660), or after 72 hr by determina-

TABLE II. Selection of Strains.

Group	Amino acid	Microorganism	Medium
I	Leucine Isoleucine Valine	*S. fecalis*	Modified Schweigert (19)
II	Argiinne Threonine	*S. fecalis*	Greenhut (20)
III	Lysine Phenylalanine Hystidine Serine Proline Glycine Aspartic acid Methionine Cystine Tyrosine Glutamic acid	*L. mesenteroides*	Barton–Wright (21)
IV	Tryptophan	*L. arabinosus*	Wooley–Sebrell (22)

tion of the lactic acid that had developed using 0.1 *N* or 0.05 *N* NaOH with bromothymol blue as indicator. Assays of hydrolyzed, pure casein were carried out simultaneously with the microbiological determination of the amino acids of the hydrolyzed enzyme.

Results. The results are given in Table III, together with a comparison of the results obtained by Bray and Malmstrom (26). As shown, satisfactory agreement was obtained in most cases. It is not known whether the rather large disagreement in the case of valine is significant.

Discussion. Various preparations of xanthine oxidase of buttermilk appear to vary somewhat with the season. Those that showed the greatest activity and purity have been selected for the analyses reported here. Previous trials were made in order to fix the best conditions of time, temperature, and concentrations of the reagents for the hydrolysis, employing monodimensional paper chromatography and by the procedure of Klungsøyr, *et al.* (25).

Control of the microbiological methods was made by assaying the amino acids of pure casein employing similar conditions.

No microbiological method for alanine was available.

All the samples in Table I were assayed by microbiological methods. The last sample listed in Table I was a pool of several batches of enzyme, and it was used to compare determinations of amino acids by both microbiological and ion-exchange procedures.

Both turbidimetry and titration of the lactic acid that had been produced were employed in the determination, but we feel data obtained by titration are more reliable, and these were the ones we recorded (Table III). It was not possible to make a sufficiently large number of assays for all of the amino

TABLE III. Amino Acid Composition of Xanthine Oxidase by Different Methods.

	Microbiological method		Spinco method		Bray analysis (26)
Amino acid	Amino acid (%)	Moles/275,000 g	Amino acid (%)	Moles/275,000 g	Moles/275,000 g
Leucine	8.20	200	7.68	187	206
Isoleucine	4.70	114	3.36	82	120
Valine	5.10	142	4.98	138	163
Arginine	6.04	106	5.60	99	105
Threonine	6.01	164	5.57	152	169
Lysine	7.20	155	6.69	144	162
Phenylalanine	6.10	114	5.62	105	118
Hystidine	2.70	54	2.72	55	55
Serine	4.90	155	4.82	152	154
Proline	4.65	132	4.64	132	130
Glycine	4.10	198	4.19	202	196
Glutamic acid	10.80	230	10.27	219	243
Aspartic acid	8.41	201	8.23	197	199
Methionine	2.08	44	1.26	26[a]	47
Tyrosine	3.70	62	4.03	86	61
Cystine	2.40	30	—	—[b]	61
Tryptophan	0.72	11	0.70[c]	10[c]	9
Alanine[d]	—	—	4.46	173	179
Total values	92.27[d]	2285	87.22[b]	2201	2377

[a] Methionine values by Spinco method on native samples were very low.

[b] Cystine without values by Spinco method (native samples).

[c] Tryptophane values were obtained only by microbiological and De Sullivan methods (23,24).

[d] Alanine without values by microbiological method. For the total figures, the values obtained by Spinco method were used.

acids, therefore, the standard error of the values is not shown.

The laboratory work and comparative data show the utility of microbiological methods in the determination of the amino acids of a hydrolized protein. Microbiological assays make it possible to determine many amino acids with the same analytical organisms and the same medium.

Nutritive media can be prepared for a number of amino acids that are sensitive to a certain strain. It is possible to choose in advance the more exacting strains with tests for strain selection.

The use of different types of hydrolysates in the microbiological assay make it possible to determine the amino acids that may be altered during one of the various hydrolyses. For example, tryptophan is destroyed by acid hydrolysis.

Summary. Highly purified xanthine oxidase was obtained from fresh, nonpasteurized buttermilk, and amino acid composition was determined by microbiological and ion-exchange (Beckman/Spinco Model 120B) methods.

The values obtained by the present methods were compared with the amino acid composition of xanthine oxidase from the same source given by Bray and Malmstrom (*26*), by the method of Moore *et al.* (*23*).

These data are a very good confirmation of Bray's work.

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