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THE USE OF A BACTERIAL L-AMINO ACID DECARBOXYLASE FOR THE CONTROL OF THE DEGREE OF RACEMIZATION OF AMINO ACID MIXTURES OBTAINED FROM PROTEIN HYDROLYSATES

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SUMMARY

A method for the simultaneous determination of D-forms of glutamic acid, lysine and arginine in protein amino acid mixtures is described. It is based upon the use of the acetone powder of an *E. coli* strain (containing L-amino acid decarboxylases which are active on these three amino acids at the same time), in conjunction with the ion-exchange amino acid chromatography. Since it has been observed that in the acid hydrolysis of proteins for the industrial preparation of amino acid mixtures, when glutamic acid, lysine and arginine are not undergoing racemization, all the other amino acids are also free of D-isomers, the authors propose that this method can be adopted for quality control of this process.

INTRODUCTION

New methods for the production of amino acid mixtures on an industrial scale, starting from acid hydrolysates of proteins of different sources, by means of a high yield ion-exchange procedure, have been recently achieved[1]. These amino acid mixtures, according to their composition (which is a function of the amino acid profile of the starting protein) may find wide application in the pharmaceutical field as well as in the nutrition of man and aninals. Moreover, this procedure, which can be applied to both homogeneous proteins and to proteinaceous materials also containing other constituents, can be considered as a valid means for better utilization of protein wastes.

The first step of the process is acid hydrolysis of the protein. According to the nature of the starting material, the concentration of sulfuric acid can be 3–9 N, whereas the protein in the hydrolysis mixture can reach the concentration of 25 % (p/v). The hydrolysis, which can be carried on either at atmospheric pressure or under high pressure, must obviously proceed as far as possible in order to get the highest possible final yield of amino acids, avoiding, on the other hand, the formation of D-stereoisomers which do not have any biological value.

Consequently, to obtain optimal results by hydrolysis of a new protein and for quality control of the amino acid mixtures produced by a working plant, the avai-

lability of a rapid and easy analytical method for the determination of the possible degree of racemization is necessary.

In our laboratories it was repeatedly observed (for different proteins and proteinaceous materials) that, if the hydrolysis conditions were not able to cause an appreciable racemization of glutamic acid, lysine and arginine, D-forms of the remainder amino acids were also not detectable. For the determination of the possible D-isomer of aspartic acid, proline, alanine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine, the amino acid mixtures were submitted to the activity of D-amino acid oxidase obtained from pig kidney, according to the methods reported by BOULANGER AND OSTEUX[2], MUELLER[3] and DIXON AND KLEPPE[4-6]. For the determination of D-glutamic acid, D-lysine, D-histidine and D-arginine, which were badly oxidized by this enzyme, the amino acid mixtures were incubated with specific L-amino acid decarboxilases of bacterial origin, as reported by GALE[7]. In both cases before and after the enzymatic treatment, the amino acids of the mixtures were determined by ion-exchange chromatography, in order to evaluate the percentage of D-forms eventually present. When using the D-amino acid oxidase as a crude acetone powder (which is preferable to the purified enzyme for its better stability), the determination of L-amino acids, present in considerable amount in the crude preparation, was to be made.

At this point it has to be mentioned that in a recent paper in which a high sensitivity method for the determination of D-amino and L-amino acids as DL- and LL-dipeptides is described, MANNING AND MOORE[8] report that in the acid hydrolysis of ribonuclease (even if carried on in a very different way and for other purposes), glutamic acid forms the D-isomer in a higher percentage (4.2 %) than threonine and serine (0.2 %). They report also that in the acid hydrolysis of synthetic bradykinin, the serine residue racemizes at a lesser extent (0.45 %) than arginine (1.7 %). Contrarily, as NEUBERGER[9] reported in 1948, it was retained that these hydroxyamino acids, owing to the hydroxyl group in β-position, were particularly prone to racemization under acidic treatment.

On the basis of these experimental data and particularly after our observations which were previously mentioned, it could be assumed that the control of the acid hydrolysis conditions for the industrial manufacturing of the amino acid mixtures is feasible by following merely the racemization degree of glutamic acid, lysine and arginine.

Since in our laboratories a strain of *E. coli* particularly rich in many L-amino acid decarboxylases has been isolated, it was possible to set up a simple and rapid method, which in conjunction with the amino acid column chromatography, allows the simultaneous determination of these three amino acids.

In this paper the preparation of the enzyme as well as the preparation of the substrate and the analytical procedure are reported. The precision of the method and the degree of sensitivity are also discussed.

MATERIALS

A strain of *E. coli* isolated in our laboratory was employed. The stock culture of the microorganism is carried on DIFCO Bacto Nutrient Agar slants. In order to obtain the inoculum for the final culture, the organism is inoculated into a few

millilitres of DIFCO Bacto Nutrient Broth and incubated for 20–24 h at 37°. The culture medium consists of 3 % DIFCO Bacto Casitone and 3 % glucose.

L-Glutamic acid, L-lysine·HCl, L-arginine, D-glutamic acid, D-lysine·HCl, D-arginine·HCl, DL-glutamic acid, DL-lysine and DL-arginine·HCl were employed. All these amino acids were purchased from Fluka.

Phosphate–citrate buffer (pH 5.2) was prepared by adding 46.6 ml of a 0.1 *M* solution of citric acid (19.2 g of citric acid per 1000 ml) to 53.6 ml of a 0.2 *M* solution of sodium orthophosphate mono acid (35.6 g of $Na_2HPO_4 \cdot 2H_2O$ per 1000 ml).

The following amino acid solutions in buffer (pH 5.2) were employed: 1 µmole of L-glutamic acid per ml; 1 µmole of L-lysine per ml; 1 µmole of L-arginine per ml; a mixture of 1 µmole of L-glutamic acid, 1 µmole of L-lysine·HCl and 1 µmole of L-arginine per ml; a mixture of 1 µmole of D-glutamic acid, 1 µmole of D-lysine·HCl and 1 µmole of D-arginine·HCl per ml; a mixture of 1 µmole of DL-glutamic acid, 1 µmole of DL-lysine and 1 µmole of DL-arginine·HCl per ml.

A solution containing 6 % sulfosalicylic acid was employed as deproteinating agent. An amino acid analyzer "Aminolyzer", manufactured by Optica Co., Milan, was used.

METHODS

1000 ml of sterile culture medium are inoculated with *E. coli* and incubated in a thermostat at 37° for 24 h. Then the cell suspension is distributed into 250-ml centrifuge bottles and centrifuged for 15 min at 4000 r.p.m.; the supernatant liquid is decanted and all the sediments, resuspended in water, are gathered into one centrifuge bottle and centrifuged down again. The cells are then suspended with 5 ml of water, then 25 ml of acetone are added at room temperature to this suspension, which is filtered under very moderate vacuum, employing a rapid-flow filter paper. Just before the precipitate is dry on the filter, it is washed with 100 ml of acetone, followed by 100 ml of ether. When all the ether is evaporated, the powder is recovered from the filter and put into a desiccator over phosphoric anhydride. Under these conditions, its activity lasts more than 2 months. From 1 l of culture, the powder yield is about 400 mg.

An amount of this powder is suspended in 1 ml of phosphate–citrate buffer. 1 ml of the amino acid solution in the same buffer is added and then the mixture is agitated for 40 min at room temperature. The reaction mixture is now centrifuged for 15 min at 4000 r.p.m.; 1 ml of the supernatant fluid is transferred into another centrifuge tube and 1 ml of the 6 % sulfosalicylic acid solution is added. After a few seconds of shaking by hand, the tube is centrifuged at 10000 r.p.m. for 10 min. 0.4 ml of the supernatant solution may then be employed for the analysis of the amino acids on each column, by using the analytical system described by MONDINO in a previous paper[10]. In order to eliminate completely the overlapping of lysine and histidine peaks, the temperature of the short column for the separation of the basic amino acids has been lowered to 48°.

Since in our analytical system, as reported previously[10–14], the peak evaluation is feasible by simply measuring the height of the peaks over the baseline in mm, from the ratio between the height of the peaks of the treated sample chromatogram and the height of the peaks of the chromatogram obtained for the untreated amino acid solution, the percentage of D-isomer possibly formed can be evaluated.

RESULTS AND DISCUSSION

Since it can be presumed that the L-glutamic acid, L-lysine and L-arginine decarboxilases are not present in the powder of our *E. coli* strain all at the same concentration level and that the optimum pH is not the same for the three enzymes, it has been necessary to check the enzymatic activity of the powder on the three amino acids separately at the same pH, with the same incubation time. According to the methodology previously described, the solutions containing 1 μmole of the single L-amino acids (L-glutamic acid, L-lysine and L-arginine) have been tested by addition of different amounts of powder. In this way the least amounts of powder necessary for obtaining the complete decarboxylation of 1 μmole of amino acid have been found. They are as follows. For the L-glutamic acid the least quantity is between 45 and 50 mg, depending upon the preparation. For the L-lysine it is between 35 and 40 mg and for the L-arginine it is less than 10 mg. In Fig. 1, the titration curves are reported. The complete decarboxylation of the solution containing the mixture of L-glutamic acid, L-lysine and L-arginine at the concentration level of 1 μmole/ml of each amino acid, was consequently achieved by employing 50 mg of acetone powder for a 40-min incubation time. The amino acid chromatogram obtained for this solution after incubation was in fact totally lacking glutamic acid, lysine and arginine. When these three amino acids are to be checked in the reciprocal presence, at different concentration levels, the amount of powder has to be chosen taking into consideration the curves reported in Fig. 1.

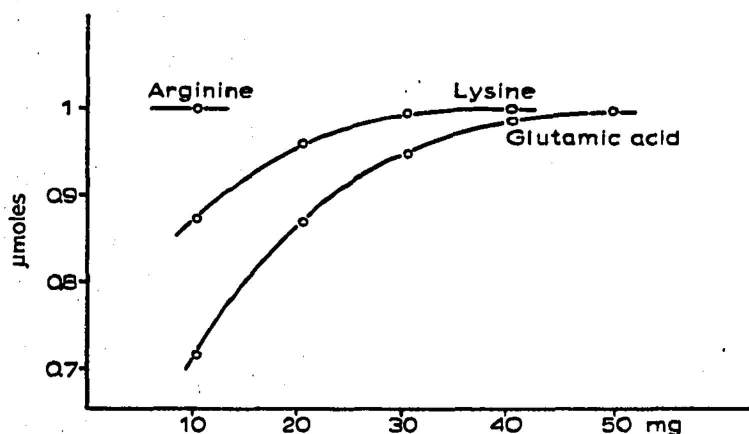

Fig. 1. Titration curves of the enzymatic activity of the three decarboxylases present in the acetone powder. On the abscissae the mg of the crude acetone powder employed are reported.

In order to check the total enzymatic inactivity of the amino acid decarboxylases produced by our strain of *E. coli* toward the D-isomers, the solutions containing 1 μmole/ml of the single D-amino acids (D-glutamic acid, D-lysine and D-arginine) have been tested according to the methodology described above. The results of this experiment, repeated three times, confirmed that these amino decarboxylases are active only toward the L-forms. Having obtained a very strict coincidence in the height of the peaks both in the chromatograms of the samples which were treated with the enzyme, incubated and deproteinated and in the chromatograms of the blanks to which only a sulfosalicylic acid solution was added, it can be stated that during the de-

proteination process no loss of glutamic acid, lysine and arginine takes place for adsorption or other causes.

In Table I are reported the results of an experiment, which was performed in order to check the degree of precision of the determination of D-isomers. The solution containing a mixture of DL-glutamic acid, DL-lysine and DL-arginine at the concentration level of 1 μmole/ml of buffer (pH 5.2) has been incubated with the acetone powder according to the method previously described. The experiment was repeated 6 times, and every time a chromatogram was run. The peak heights of the remaining D-forms of glutamic acid, lysine and arginine are reported and their values, statistically evaluated, confirm a precision degree better than $\pm$ 1.6 %.

TABLE I

RESULTS OF THE CHROMATOGRAMS OF A MIXTURE OF DL-ISOMERS AFTER TREATMENT WITH THE *E. coli* ACETONE POWDER

The peak heights of the remaining D-isomers are expressed in mm.

Chromatogram	*1*	*2*	*3*	*4*	*5*	*6*	*S.D.*	*C.V.*
D-Glutamic acid	57.5	56.0	55.0	56.5	56.0	57.0	±0.87	±1.55
D-Lysine	99.5	97.5	97.0	99.0	97.7	99.5	±1.21	±1.23
D-Arginine	33.0	33.0	33.5	34.5	33.0	34.0	±0.49	±1.47

As far as the sensitivity is concerned, it can be said that it is strictly depending upon the degree of sensitivity offered by the method of amino acid chromatography employed. Moreover, a way of enhancing the analytical sensitivity for these three amino acids (which in this case are usually present in a very small amount), consists of charging a larger sample on the chromatographic column, even with the chance of having all the other amino acid peaks off scale. Consequently, a method of amino acid analysis has to be adopted in which the peaks of glutamic acid, lysine and arginine are well separated down to the baseline and do not overlap at all with the neighboring peaks. This occurs obviously when a complete mixture of amino acids is submitted to the action of the powder. As a matter of fact the overloading on the column of all the other amino acids, which are not decarboxylated, will cause a broadening of their peaks that can interfere with the small peaks corresponding to the D-forms, if any, of glutamic acid, lysine and arginine. Operating in this way and with the help of the sensitivity range expander which can be found on our apparatus[10], two parts of D-glutamic acid and two parts of D-lysine can be detected in the presence of 1000 parts of the L-isomers; concerning D-arginine the sensitivity is somewhat lower, owing to the form of this peak.

For readers desiring to try the method described, agar slants of the *E. coli* employed can be obtained from the authors.

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