# L-Aspartic Acid Derivatives as Precursors of Aspartyl-dipeptide Sweeteners

## I. Synthesis of L-aspartic acid by biocatalytical reductive amination of fumaric acid

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*L-aspartic acid and its N-substituted derivatives are used as carboxylic components of dipeptides in the research of molecular bases of sweet taste dipeptide ligands. This work refers to experiments performed in order to obtain L-aspartic acid by stereo selective transformation of fumaric acid in enzymatic catalysis. The biocatalyst used for this purpose was obtained by immobilization of E. Coli cells having high aspartase activity. The most active immobilized cells were obtained by the entrapment in cross linked polyacrylamide gel method and additional treatment with glutardialdehyde, for which important parameters, like concentration of monomer, cross linking reagent and glutardialdehyde were studied. Kinetics of enzymatic reaction with immobilized biocatalyst was studied. Conditions for the enzymatic conversion of fumaric acid into L-aspartic acid using immobilized active cells were also investigated.*

*Keywords: microbial aspartase, immobilization, enzymatic reaction, L-aspartic acid*

Low calories sweeteners are sugar substitutes that offer people with diabetes the possibility to control their total carbohydrate intake and allow them to satisfy their taste for sweets without affecting blood glucose level. In addition, low-calories sweeteners are effective for other purposes too: weight maintenance, weight reduction, reduction of dental caries, and reduction in the risks associated with obesity.

Although a large number of sweet testing substances are known, their use as sweeteners is limited by toxicological, technological and economical barriers. The fact that none of the intensive sweeteners available at present gathers the qualities of the ideal sweetener (complete lack of toxicity, stability in a large domain of temperature and *p*H) justifies the continuous research in this area.

The serendipitous discovery of the sweet taste of L-α-aspartyl-L-phenylalanine-1 methyl ester (aspartame) pointed out the sweetness potential of aspartyl-dipeptide derivatives. Recent studies of molecular biology, referring to sweet taste protein receptors, revealed the fact that among the dipeptide sweet taste ligands, some N-substituted aspartame analogues may have a sweetening power of 1.000 – 50.000 times greater than sucrose (1).

The importance of L-aspartic acid results from its use as the carboxylic component of dipeptide derivatives above mentioned as for the synthesis of many pharmaceutical products and biological reagents (2). This work refers to the obtaining of this amino - acid by reductive amination of fumaric acid in enzymatic catalysis, method superior to chemical synthesis due to the stereo selective action of a specific enzyme, aspartase (3).

## Experimental part

A microbial biomass having L-aspartate-ammonium-lyase (aspartase) activity was used to obtain an enzymatic catalyst for the reductive amination of fumaric acid. This active biomass was formed by submerse cultivation of a strain of *E. coli* (*E. coli* ICCF 25), from the culture collection of the National Institute of Chemical-Pharmaceutical Research & Development. Cell growth and aspartase production occurred in an inexpensive nutrient medium containing an assimilable source of carbon, nitrogen and mineral salts, under aerobic conditions. Microbial cells containing aspartase were collected by centrifugation and washed to remove most of the growth medium.

Taking into account that *E. coli* aspartase is an intracellular enzyme and that it is cost-effective to use the biocatalyst continuously or in repeated reaction cycles, we studied the immobilization of aspartase, as it is in *E. coli* biomass (4).

The biomass used in immobilization experiments was characterized by water content and aspartase activity.

Aspartase activity of native or immobilized biomass, was determined according to a standard assay carried out as follows: a reaction mixture containing 0.1 g of native biomass (or the correspondent quantity of immobilized biomass) and 5 mL of 1M ammonium fumarate (adjusted to *p*H 8.5 – 9.5 with ammonia, was incubated at 37°C for one hour, with shaking. The reaction was stopped by immersion in a boiling water bath for five minutes and the solid material was removed by centrifugation. L-aspartic acid produced in the enzymatic reactions was measured by a spectrophotometric method based on ninhydrin reaction, after paper chromatography.
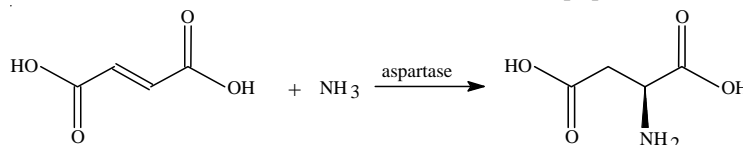
Fig. 1. The influence of acrylamide / BIS ratio on aspartase immobilization



——■—— Immobilized actitivity (μmoles/g)
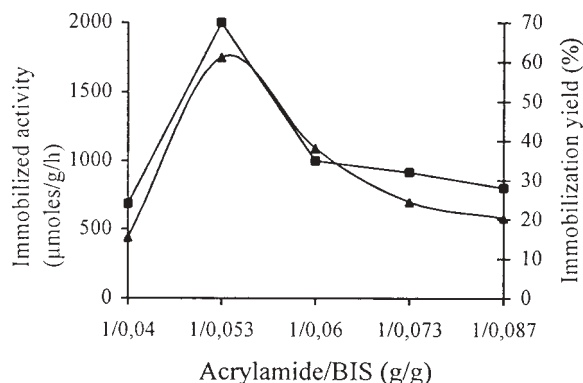——▲—— Immobilization yield (%)

Fig. 2. The influence of biomass/acrylamide ratio on aspartase immobilization

Enzyme activity was expressed as micromoles of L-aspartic acid produced by one mass unit (g) of enzyme preparation per hour.

The immobilization of *E. coli* cells with aspartase activity was carried out by the entrapment method (5). Polyacrylamide cross linked with N,N'-methylene-bis-acrylamide was used as a polymer matrix. *E. coli* cells were suspended in physiological saline. The acrylamide monomer (corresponding to a final concentration of 25% w/v) and the cross-linking reagent were added to this suspension and mixed at +8$^0$C. A solution of 5 % 1.2-bis(dimethylamino)-ethan (TEMED) and one of 2.5% w/v potassium persulfate were added and the reaction mixture was allowed to stand at 25$^0$C for 15-30 min to obtain a stiff gel. The compact gel weighted 6 fold more than the native biomass. A 10 fold activation of the aspartase from the immobilized cells was achieved by maintaining them for 32 hours, without stirring, in 1 M ammonium fumarate solution, $p$H = 9, containing 1mM Mg$^+$, at room temperature. After activation, the gel was triturated and washed with distilled water.

Kinetics of enzymatic reaction with immobilized cells was investigated by measuring the reaction rate at various values of substrate concentration, $p$H and temperature. The reaction rate was expressed as micromoles of L-aspartic acid formed per minute and gram of enzyme and it was calculated based on L-aspartic acid determination after 15 minutes of reaction.

A batch process was carried out for the enzymatic conversion of fumaric acid to L-aspartic acid using active immobilized cells. The reaction mixture was prepared by suspending immobilized active cells in a solution of ammonium fumarate containing Mg$^{2+}$ as enzyme activator and having an ammonia excess corresponding to a range of $p$H between 8.5 and 9. The reaction mixture was kept under continuous, gentle stirring, until the concentration of L-aspartic acid remained constant. At the end of the enzymatic reaction, the biocatalyst was recovered by filtration and washed with distilled water. L-aspartic acid was isolated from the bioconversion medium by precipitation at the isoelectric point, with 2N HCl solution.

Stability of active immobilized cells was investigated by measuring the remaining activity after each reaction cycle, performed at different values of temperature. In order to obtain a biocatalyst with improved operational stability, a treatment with glutardialdehyde (6), as a second cross linking reagent, was carried out during the active cell immobilization.
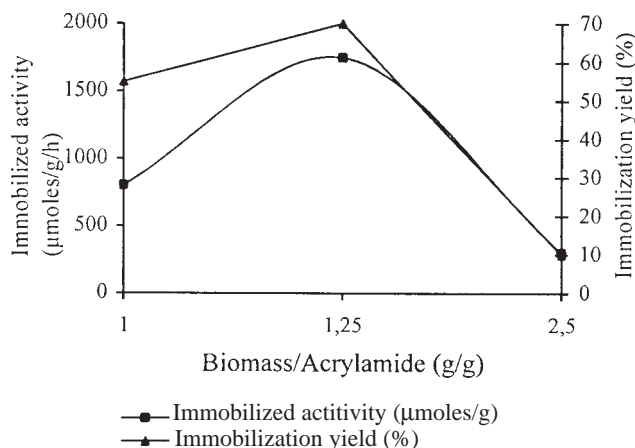
## Results and discussion

### *Immobilization of E. coli cells containing aspartase by polyacrylamide gel entrapment method*

In order to achieve the best conditions for the immobilization of aspartase containing *E. coli* cells in cross linked polyacrylamide gel, we studied two important parameters that affect this procedure: relative ratio of monomer and N,N'-methylene bisacrylamide (BIS) (fig. 1) and relative ratio of biomass and monomer amounts (fig. 2).

As it can be seen in figures 1 and 2, the best results (immobilized cell activity =1750 μmoles/g/h and immobilization yield = 70%) were obtained at a ratio = 1/0.053 of monomer to bifunctional reagent, in connection with a ratio = 1:1.25 of cells (wet weight) to monomer. In these conditions, the cells are entrapped in the lattice formed during the polymerization reaction, retaining most of their enzymatic activity.

### *Kinetics of reductive amination reaction of fumaric acid using immobilized aspartase*

The behavior of immobilized aspartase in the reductive amination of fumaric acid was studied in different reaction conditions, shown in figures 3 -5.

In order to establish the optimum value of substrate concentration, 6 g of immobilized enzyme were suspended in 100 mL ammonium fumarate solution of concentration varying between 0.25 and 1.5 M, containing 1mM Mg$^{2+}$ and an ammonia excess corresponding to $p$H = 9. Carrying out the reaction at 37$^0$C, with gentle stirring, the maximum reaction rate was obtained with 1M ammonium fumarate solution, as it can be seen in figure 3.
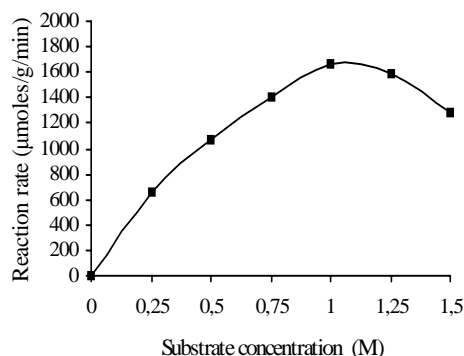


Fig. 3 The dependence of reaction rate upon substrate concentration

To point out the influence of temperature and pH, 6 g of immobilized enzyme were stirred with 100 mL 1M ammonium fumarate containing 1mM $Mg^{2+}$ and an ammonia excess corresponding to $p$H = 9 and the reactions were performed at different temperatures; then, the same concentrations of enzyme and substrate were applied in other reactions, at $40^0$C, varying the $p$H between 6 and 10.
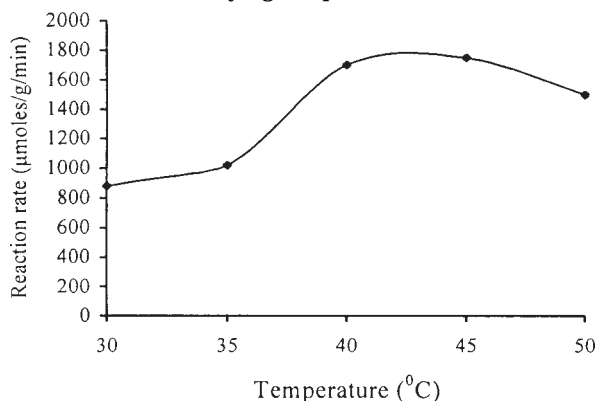


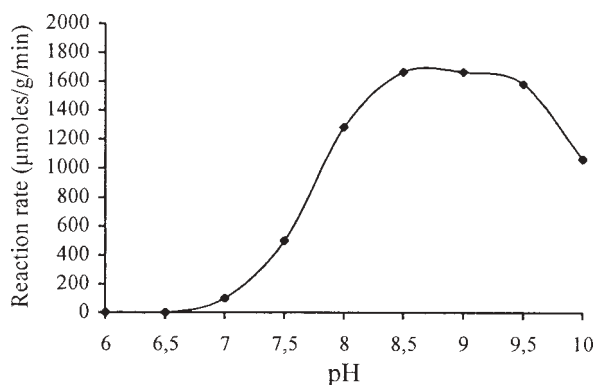Fig. 4. The dependence of reaction rate upon temperature
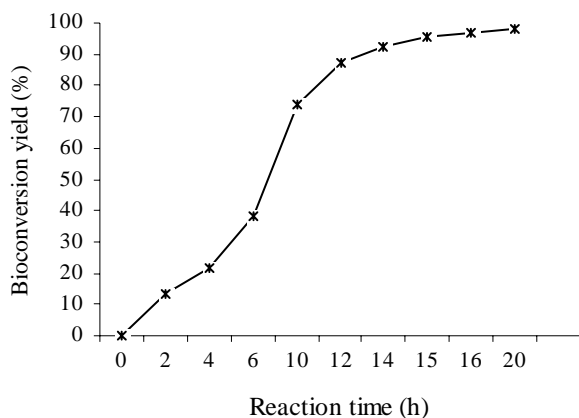


Fig. 5. $p$H dependence of reaction rate



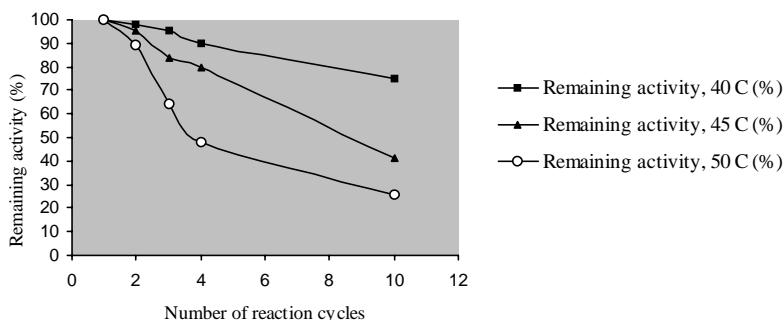Fig. 6. Time course of bioconversion yield



Fig. 7 Operational stability of biocatalyst immobilized by entrapment in cross linked polyacrylamide

According with the results displayed in figures 4 and 5, the maximum reaction rate of 1667 μmoles L-Asp/g biocatalyst/min. was obtained by the action of 6 g immobilized enzyme on 100 mL 1 M ammonium fumarate solution of $p$H = 8.5 − 9, at 40 − $45^0$C.

*Production of L-aspartic acid by bioconversion of fumaric acid*

L-aspartic biosynthesis was carried out using 60 g of immobilized *E. coli* cells with aspartase activity, 1000 mL 1 M ammonium fumarate solution containing 1mM $Mg^{2+}$ and ammonia, $p$H = 9, at $40^0$C, under gentle stirring (150 rpm). According to the experimental results (fig. 6), the bioconversion yield was practically quantitative (98%) after 20 h of enzymatic reaction.

Pure L-aspartic acid was isolated (85 % yield) after removing the biocatalyst, by precipitation at isoelectric point ($p$H$_i$ = 2.7) with 2N HCl.

*Operational stability of immobilized aspartase*

The stability of immobilized active cells was also investigated by reusing the immobilized biocatalyst in a number of reaction cycles, at different values of temperatures. As shown in figure 7, the remaining activity of the biocatalyst decreased after a number of reaction cycles at any of the temperatures tested.

By adding a 25% glutardialdehyde solution in the polymerization mixture, during the active cell immobilization, an improvement of the immobilization yield as well as of the biocatalyst stability was obtained, as shown in tables 1 and 2.

**Table 1**

GLUTARDIALDEHYDE INFLUENCE ON THE IMMOBILIZATION YIELD BY ENTRAPMENT IN CROSS LINKED POLYACRYLAMIDE GEL

| Exp.no | Glutaraldyehide/ biomass | Immobilization yield (%) |
|--------|--------------------------|--------------------------|
| 1. | 1:20 | 75 |
| 2. | 1:15 | 72 |
| 3. | 1:12 | 70 |
| 4. | 1:10 | 68 |
| 5. | 1:8 | 65 |

**Table 2**

STABILITY IMPROVEMENT OF ACTIVE CELLS IMMOBILIZED BY ENTRAPMENT IN CROSS LINKED POLYACRYLAMIDE GEL AND ADDITIONAL TREATMENT WITH GLUTARDIALDEHYDE

| Number of reaction cycles | Remaining activity (%) |
|---------------------------|------------------------|
| 1 | 98 |
| 2 | 97 |
| 3 | 95 |
| 4 | 95 |
| 5 | 95 |
| 10 | 95 |
| 15 | 95 |
| 20 | 95 |
| 50 | 90 |

The results in table 1 were obtained using for the polymerization *in situ* the optimum conditions pointed out in figures 1 and 2.

The biocatalyst was used in reactions carried out at $40^0$C, according to the optimum conditions pointed out in figures 3 – 6.

## Conclusions

Aspartase produced by *E. coli* ICCF 25 can be immobilized as whole microbial cells by entrapping in cross linked polyacrylamide gel and additional treatment with glutardialdehyde, with an immobilization yield of 75 %.

The biocatalyst obtained by immobilization can be used for the stereo selective reductive amination of fumaric acid with 98% yield, in the following conditions: substrate concentration: 1M containing 1mM $Mg^{2+}$, enzyme concentration: $10^5$ aspartase activity units/ L substrate solution, $p$H = 9, temperature: $40^0$C, stirring: 120 rpm, time: 20 hours.

The remaining activity of the immobilized biocatalyst, corresponding to a conversion yield (L-aspartic acid synthesis) of 98 – 90 %, during 50 reaction cycles, indicates the immobilized cells to be an efficient biocatalyst for batch or continuous production of L-aspartic acid.

## References

1. GOODMAN, J. R., AMINO, Y., BENEDETTI, E., Pure Appl. Chem., **74,** Nr. 7, 2002, p. 1101
2. KAWASAKI, K., YAMASHIRO, Y., YAMAMOTO, S., Chem. Pharm. Bull., **43,** 1995, p.2133
3. SHI , W., Biochemistry, **36**, 1997, p. 9136
4. KATCHALSKY-KATZIR, E., Trends in Biotechnol., **11**, 1993, p.471
5. SKRYABIN, G. K., KOSCHEENKO, K. A., Methods Enzymol., **135,** 1987, p.198
6. BENHAR, I., WANG, Q., J. Biol. Chem., **269**, 1994, p.1339