PREPARATION OF ^{35}S-LABELLED L-METHIONINE AND L-CYSTINE BY BIOSYNTHESIS

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S u m m a r y

The modified method for biosynthesis of L-methionine-^{35}S and L-cystine-^{35}S from baker's yeast (Saccharomyces cerevisiae) is described. The yeast was cultivated in a sulphur-deplated medium containing carrier-free $Na_2{}^{35}SO_4$, under air bubbling during 24 hours. Yeast proteins were hydrolyzed enzymatically into free amino acids, and L-methionine-^{35}S and L-cystine-^{35}S were isolated by ion-exchange chromatography on a column. Radioactive yields for L-methionine-^{35}S and L-cystine-^{35}S were 30% and 10%, respectively. The obtained products have high specific activity,1-10 Ci/mmol, and radiochemical purity better than 95%.

Key words: L-methionine-^{35}S, L-cystine-^{35}S, biosynthesis, yeast

I N T R O D U C T I O N

Methionine is an essential amino acid which has an important role in biological investigations owing to its terminal SCH_3 group. Incorporation of radioactive sulphur into the methionine molecule makes

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it possible to get an insight into the mechanism of biological transformation of this amino acid.

^{35}S-Labelled methionine can be obtained by direct chemical synthesis or biosynthesis in the organism where thioamino acids are formed from inorganic sulphur.

The aim of our work was to obtain exclusively the L-isomer of the mentioned amino acid of high specific activity, of the order Ci/mmol, and of high radiochemical purity what was possible to achieve by the biosynthetic method.

In the literature are described several methods for the biosynthesis of L-methionine-^{35}S, which differ by microorganisms used, the hydrolysis procedure, or the method of separation and isolation.

The microorganism most often used in the biosynthesis of thioamino acids were baker's yeast (Saccharomyces cerevisiae) or Saccharomyces carlbergensis[1,3,4,7,8,9] but in the literature are also mentioned Torula utilis[2] and bacteria Escherichia Coli[5,6]. By growing yeast or Escherichia Coli in a suitable medium in the presence of inorganic ions, $Na_2{}^{35}SO_4$ or $(NH_4)_2{}^{35}SO_4$, labelled yeast proteins are formed and decomposed by hydrolysis into free amino acids, L-methionine-^{35}S and L-cystine-^{35}S.

For hydrolysis of proteins most often acid conditions are used, but hydrolysis can be carried out by enzymes[7]. Isolation and separation of labelled amino acids from hydrolysates is performed in several ways i.e., by preparative paper chromatography[5], paper electrophoresis[10], ion-exchange chromatography on a column[6,11] or by combined crystallization[3].

In the present paper a modified method for biosynthesis of L-methionine and L-cystine labelled with ^{35}S is described. Baker's yeast was cultivated in low-sulphur medium according to Williams and Dawson[3]. By varying the quantity of the inorganic sulphate ions, used as a carrier, we found the optimum concentration at which ^{35}S is almost quantitatively incorporated into yeast proteins. Radioactive labelling was performed with carrier-free $Na_2{}^{35}SO_4$[12] produced in the Institute "Boris Kidrič" Radioisotope Laboratory. Yeast proteins

were hydrolyzed enzymatically by means of pronase (Streptomyces griseus protease) and 80-85% of initial radioactivity was obtained in the hydrolysate. L-Methionine-^{35}S and L-cystine-^{35}S were isolated from hydrolysate by ion-exchange chromatography on a column.

L-Methionine-^{35}S and L-cystine-^{35}S, obtained in yields of 30% and 10% respectively, have radiochemical purity >95% and specific activity 1-10 Ci/mmol.

E X P E R I M E N T A L

Growth of yeast

To the sterile medium (one liter) carrier-free $Na_2{}^{35}SO_4$, (0.1--1 Ci), was added, than the pH was adjusted to 5 by using H_3PO_4 and the solution obtained was poured into the fermentation vessel (Fig.1). One liter of the medium contained the following components:

Glucose	20.0	g
$(NH_4)_2HPO_4$	3.5	g
KH_2PO_4	0.2	g
$MgSO_4.7H_2O$	0.123	g
$MgCl_2.6H_2O$	0.101	g
Zinc acetate	0.4	mg
$FeCl_3$	0.15	mg
$CuCl_2$	0.025	mg
Citric acid, trisodium	1.0	g
L-Asparagine, monohydrate	2.5	g
Biotine	0.01	mg
Pantothenic acid, calcium salt	0.5	mg
Inositol	10.0	mg
Thiamine	0.6	mg
Pyridoxine	1.0	mg

After inoculation with 300 mg of fresh baker's yeast, growth was carried out at 37°C under air bubbling. After 24 hours, the yeast was harvested by centrifugation. In the case when less than 70% of the starting radioactivity was incorporated into the yeast cells, in the supernatant 10 g of glucose and 100 mg of yeast were added and cultivation was prolonged for another 24 hours. In this way 90-95% of radioactivity was incorporated into the yeast cells. After centrifugation, the yeast was washed with water, dehydrated by absolute ethanol and degreased by refluxing with 50 ml of n-hexane for 30 minutes.

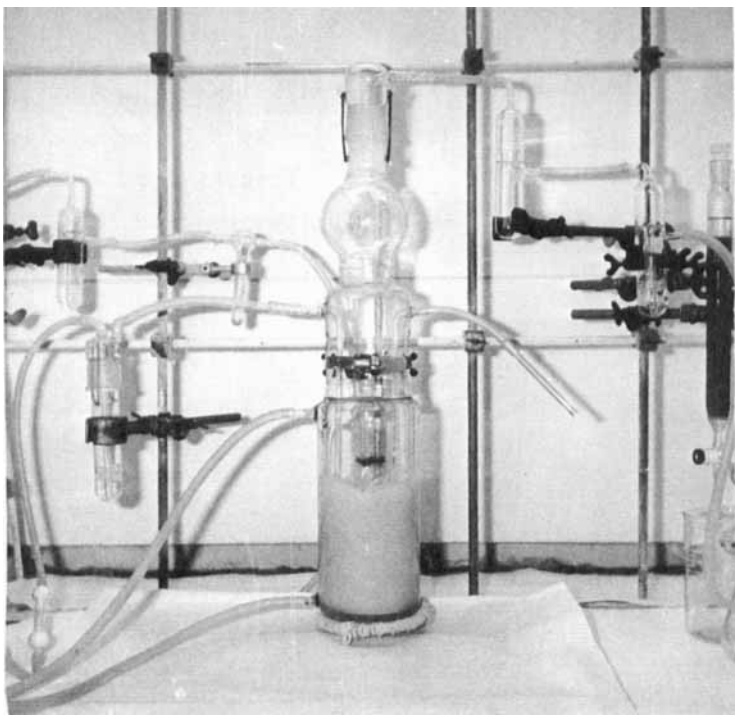

Fig. 1. The fermentation vessel

Hydrolysis and isolation

Yeast proteins were hydrolyzed with pronase (Calbiochem) at 37°C in 0.033 M phosphate buffer at pH 7.4 (250 ml of buffer, 125 mg of pronase and 10 mg of chloramphenicol).

After hydrolysis for 24-48 hours, the suspension was centrifuged and the supernatant was passed through the ion-exchange column (25x200 mm) of Dowex 50Wx8, 200-400 mesh, in H^+ form. The column was washed with water and total radioactivity was eluted with 1 N ammonia at the flow rate of 2.5 ml/min. The radioactive eluate was evaporated to dryness under reduced pressure, the residue was dissolved in water, acidified to pH 2 by using 10% HCl and the solution obtained was passed through the second ion-exchange column (25x200 mm) of Dowex 50Wx8, 200-400 mesh, in H^+ form. The column was washed with water, and L-methionine-HCl-^{35}S was eluted with 1.1 N HCl at the flow rate of 1.5 ml/min in nitrogen stream. Portions of 15 ml each were collected. L-Methionine-^{35}S appeared in the eluate between 500-700 ml.

Fractions of L-methionine-HCl-^{35}S of radiochemical purity >95% were combined and evaporated to dryness under reduced pressure. The residue was dissolved in water and the solution was passed through an anion-exchange column, (25x200 mm) with Dowex 2x8, 50-100 mesh, in acetate form, and L-methionine-^{35}S was eluted with water.

Accompanying radiochemical impurities were methionine sulphoxide-^{35}S and methionine sulphone-^{35}S. The preparation also contained inactive amino acids L-valine and L-leucine.

In order to minimize auto-oxidation, the L-methionine-^{35}S solution of the specific activity 4-5 mCi/ml was stored at -20°C.

To elute L-cystine-^{35}S, the concentration of hydrochloric acid was increased to 2.5 N. L-Cystine-^{35}S appeared in fractions with 300-500 ml after the change in eluting acid.

Radiochemical purity was checked by using descending paper chromatography on Whatman No. 1 paper in the following solvent sy-

stems: n-butanol/acetic acid/water (4:1:1) and chloroform/methanol/NH_4OH conc./water (2:2:0.57:0.40) and by using thin-layer chromatography on Silica Gel G (Merck), on glass plates (50x200 mm), 0.25 mm thickness layer, in the developing solvent systems: n-butanol/acetic acid/water (4:1:1) and ethanol/water (70:30).

Radioactive compounds were detected by using Berthold-Dünnschicht und Papierchromatogramm Scanner LB-280.

Radiochemical purities of L-methionine-^{35}S and L-cystine-^{35}S were >95%. Biochemical analysis of both products applied on experimental animals gave satisfactory results.

RESULTS AND DISCUSSION

On the basis of the results obtained we can draw the following conclusions:

- The specific activity of L-methionine-^{35}S and L-cystine^{35}S strongly depends on the activity level of $Na_2{}^{35}SO_4$ incorporated into the growth medium as well as on the amount of inorganic sulphate ions*.

- The optimum radioactive yield can be obtained by growing yeast in a medium which contained up to 0.5 mmol $MgSO_4$. In this case 95-98% of the starting radioactivity was incorporated into yeast proteins in 24 hours only. Growing in the medium with 1 mmol $MgSO_4$ gave in 48 hours a maximum radioactive yield of 80%.

- By applying the enzymatic hydrolysis of yeast proteins, in 24 hours up to 95% of labelled amino acids was released. The colourless hydrolysate did not destroy the ion-exchange resin, which after regeneration can be used several times. In our previous experiments proteins were hydrolyzed with 6N HCl in 24 hours and the radioactive yield obtained was up to 60%.

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