This invention relates to a process for the preparation of purified penicillin G acylase. This invention particularly relates to the purification of crude enzyme extract and to the purification of industrially important enzyme such as Penicillin G acylase. More particularly it relates to the process for the purification of penicillin G acylase from enzyme extract of Escherichia coli using macroporous beaded crosslinked copolymers. The purification achieved by the process of this invention is in high yields and is effected in a single operation.

Penicillin G acylase finds applications as a biocatalyst in the preparation of 6-aminopenicillanic acid (6-APA) and 7-amino desacetoxycephalosporanic acid (7-ADCA). 6-APA and 7-ADCA are further processed for the production of semisynthetic penicillins and cephalosporins those are potent antimicrobial agents. These applications are developed due to absolute specificity of penicillin G acylase in hydrolysing linear amide bond present in penicillins and cephalosporins to form corresponding beta-lactam nuclei.

Penicillin G acylase is used as an immobilized enzyme preparation. The protocol for preparation of immobilised penicillin G acylase constitutes fermentation of a microorganism producing penicillin G acylase (usualy Escherichia coli), extraction of the penicillin G acylase from the cells into aqueous extract, purification of the penicillin G acylase and immobilisation of the purified enzyme onto a solid support. The specific activity of the immobilised penicillin G acylase is determined by the degree of purity of the enzyme used for immobilisation. Thus, purification of penicillin G acylase is an important operation in the production of immobilised penicillin G acylase.

Different protocols are developed for purification of penicillin G acylase involves use of ion exchange, hydrophobic interactions and/or affinity chromatography either individually or in various combinations as described in Applied Biochemistry and Biotechnology vol.9, p. 573, 1984. Sepharose (agarose) activated with cyanogen bromide and derivatised with some amino acids, aromatic amines, aliphatic amines, ampicillin and amoxycillin are used for purification of penicillin G acylase as described in Applied Biochemistry and Biotechnology, vol. 9, p. 421, 1984; Biotechnology Letters, vol. 9, p. 539, 1987 and Hindustan Antibiotics Bulletin, vol. 31, p. 25, 1989.

These protocols for purification of penicillin G acylase suffer from one or more of the following disadvantages; use of column chromatography procedures, bed compression, attrition of the chromatography support when used in batch mode.

In our copending application No. NF 309/00, we have described and claimed a process for the preparation of macroporous beaded crosslinked copolymers useful as affinity chromatography supports.

The main object of the present invention is to provide a process for the preparation of purified penicillin G acylase.

Another object of the present invention is to provide a process for the purification of penicillin G acylase from enzyme extract of Escherichia coli using derivatised macroporous beaded crosslinked copolymers.

Yet another object of the invention is to develop a process, which is simple, rapid and does not involve many steps.

Still another object is to provide a process by which purified penicillin G acylase preparation is useful for its immobilization into solid supports.

Accordingly, the present invention provides a process for the preparation of purified penicillin G acylase, which comprises suspending the novel macroporous beaded crossiinked copolymers such as herein described in a crude penicillin G acylase enzyme extract prepared in a buffer solution having a concentration in the range of 2 to 20% and pH in the range of 7 to 8, agitating the suspension for a period upto 30 minutes at a temperature in the range of 20 to 30°C at an rpm in the range of 75 to 200, filtering the suspension, washing the beads with buffer having concentration in the range of 2 to 20% and pH in the range of 7 to 8, eluting the adsorbed penicillin G acylase by washing with the buffer as used above containing an organic acid or a glycol and recovering the purified penicillin G acylase by conventional methods.

In an embodiment of the invention, the novel macroporous beaded crossiinked copolymers are prepared as described and claimed in our copending application No.961/Del/2000.

In another embodiment of the invention, the buffer solution is prepared from phosphate or tris chloride and preferably has a molarity of 0.05 and a pH of 7.5.

In yet another embodiment of the present invention, the crude penicillin G acylase may be prepared from Escherichia coli cells, Bacillus megaterium, Bovista plumbea, Achrobacter sp.

In still another embodiment of the present invention, the organic acid used may be such as phenyl acetic acid,/ethylene glycol, propylene glycol.

The dialysis was effected by suspending the penicilling G acylase solution in a dialysis bag in a buffer of phosphate or tris chloride having a molarity of 0.05 to 0.2 and pH in the range of 7.00 to 8.00

Penicillin G acylase and total protein content in the enzyme extract used was 5 IU/ml and 2 mg/ml, respectively. Thus, the specific activity of penicillin G acylase in the enzyme extract was 2.5 IU/mg. The enzyme activity was determined using penicillin G as substrate as described in Hindustan Antibiotics Bulletin Vol. 20, p. 80, 1978. Protein was determined as described in Journal of Biological Chemistry vol. 193, pp.265, 1951.

The process of the present invention is described with reference to following examples, which are given by way of illustration only and should not construed to limit the scope of the invention in any manner.

Examples for the preparation of macroporous beaded crosslinked copolymers

Example 1

In an inert atmosphere of nitrogen, 1.5 grams of poly vinyl pyrrolidone was dissolved in

150 ml of distilled water in a 250 ml capacity glass reactor. 9.72 grams of allyl glycidyl ether, 8.38 grams of pentaerythritol triacrylate and 22.5 ml of cyclohexanol were added to the aqueous solution of poly vinyl pyrrolidone. The suspension was stirred at 300 rpm. 0.6 gram of azo bis isobutyronitrile was added to this suspension and the resultant polymerization reaction mixture was heated with agitation at a temperature of 70°C for 3 hours. The copolymer separated out as beads during the polymerization. The copolymer beads were isolated by filtration, washed with distilled water, followed by washing by methanol and dried in a vacuum oven at 40°C. The yield of copolymer obtained was 17.10 grams.

Example 2

In an inert atmosphere of nitrogen, 1.5 grams of poly vinyl pyrrolidone was dissolved in 150 ml of distilled water in a 250 ml capacity glass reactor. 4.30 grams of allyl glycidyl ether, 14.50 grams of pentaerythritol trimethacrylate and 22.0 ml of cyclohexanol were added to the aqueous solution of poly vinyl pyrrolidone. The suspension was stirred at 300 rpm. 0.6 gram of azo bis isobutyronitrile was added to this suspension and the resultant polymerization reaction mixture was heated with agitation at a temperature of 70°C for 3 hours. The copolymer separated out as beads during the polymerization. The copolymer beads were isolated by filtration, washed with distilled water, followed by washing by methanol and dried in a vacuum oven at 40°C. The yield of copolymer obtained was 14.5 grams.

Example 3

In an inert atmosphere of nitrogen, 2.0 grams of poly vinyl acetate was dissolved in 150 ml of distilled water in a 250 ml capacity glass reactor. 8.6 grams of allyl glycidyl ether, 7.8 grams of divinyl benzene and 23 ml of cyclohexanol were added to the aqueous solution of poly vinyl

pyrrolidone. The suspension was stirred at 300 rpm. 0.8 gram of azo bis isobutyronitrile was added to this suspension and the resultant polymerization reaction mixture was heated with agitation at a temperature of 70°C for 3 hours. The copolymer separated out as beads during the polymerization. The copolymer beads were isolated by filtration, washed with distilled water, followed by washing by ethanol and dried in a vacuum oven at 50°C. The yield of copolymer obtained was 14.25 grams.

Example 4

In an inert atmosphere of nitrogen, 1.8 grams of poly vinyl alcohol was dissolved in 150 ml of distilled water in a 250 ml capacity glass reactor. 9.0 grams of allyl glycidyl ether, 8 grams of ethylene glycol dimethacrylate and 43.5 ml of cyclohexanol were added to the aqueous solution of poly vinyl pyrrolidone. The suspension was stirred at 300 rpm. 0.6 gram of methyl ethyl ketone peroxide was added to this suspension and the resultant polymerization reaction mixture was heated with agitation at a temperature of 60°C for 4 hours. The copolymer separated out as beads during the polymerization. The copolymer beads were isolated by filtration, washed with distilled water, followed by washing by ethanol and dried in a vacuum oven at 40°C. The yield of copolymer obtained was 15.25 grams.

Example 5

In an inert atmosphere of nitrogen, 1.5 grams of poly vinyl pyrrolidone was dissolved in 150 ml of distilled water in a 250 ml capacity glass reactor. 8.5 grams of allyl glycidyl ether, 8.25 grams of trimethylol propane triacrylate and 43.5 ml of cyclohexanol were added to the aqueous solution of poly vinyl pyrrolidone. The suspension was stirred at 300 rpm. 0.5 gram of benzoyl peroxide was added to this suspension and the resultant polymerization reaction mixture was heated

with agitation at a temperature of 70°C for 3 hours. The copolymer separated out as beads during the polymerization. The copolymer beads were isolated by filtration, washed with distilled water, followed by washing by methanol and dried in a vacuum oven at 40°C. The yield of copolymer obtained was 15.35 grams.

Example 6

In an inert atmosphere of nitrogen, 2.25 grams of sodium salt of poly acrylic acid was dissolved in 150 ml of distilled water in a 250 ml capacity glass reactor. 9.0 grams of allyl glycidyl ether, 8.75 grams of trimethylol propane trimethacrylate and 43.5 ml of cyclohexanol were added to the aqueous solution of poly vinyl pyrrolidone. The suspension was stirred at 300 rpm. 0.6 gram of benzoyl peroxide was added to this suspension and the resultant polymerization reaction mixture was heated with agitation at a temperature of 80°C for 2 hours. The copolymer separated out as beads during the polymerization. The copolymer beads were isolated by filtration, washed with distilled water, followed by washing by ethanol and dried in a vacuum oven at 50°C. The yield of copolymer obtained was 15.75 grams.

Examples for the derivatisation of macroporous beaded crosslinked copolymers

Example 7

10 g of the macroporous beaded crosslinked copolymer prepared as described in Example 4 was suspended in 60 ml of ligand solution containing 34.4 g of butylamine dissolved in ethanol. The suspension was incubated in a stoppered 250-ml capacity conical flask on a rotary shaker at 100 rpm, 25°C for 48 hours. The beads were collected by filtration on a Buchner funnel, washed with 200 ml of distilled water and dried at 40°C under vacuum. The derivatised macroporous beaded crosslinked copolymer beads were stored at 4°C.

Example 8

10 g of the macroporous beaded crosslinked copolymer prepared as described in Example 4 was suspended in 60 ml of ligand solution containing 23.8 g of hexylamine dissolved in methanol. The suspension was incubated in a stoppered 250 ml capacity conical flask on a rotary shaker at 100 rpm, 25°C for 48 hours. The beads were collected by filtration on a Buchner funnel, washed with 200 ml of distilled water and dried at 40°C under vacuum. The derivatised macroporous beaded crosslinked copolymer beads were stored at 4°C.

Example 9

10 g of the macroporous beaded crosslinked copolymer prepared as described in Example 4 was suspended in 60 ml of ligand solution containing 30.4 g of octylamine dissolved in ethanol. The suspension was incubated in a stoppered 250 ml capacity conical flask on a rotary shaker at 100 rpm, 25°C for 48 hours. The beads were collected by filtration on a Buchner funnel, washed with 200 ml of distilled water and dried at 40°C under vacuum. The derivatised macroporous beaded crosslinked copolymer beads were stored at 4°C.

Example 10

10 g of the macroporous beaded crosslinked copolymer prepared as described in Example 4 was suspended in 60 ml of ligand solution containing 43.6 g of dodecylamine dissolved in methanol. The suspension was incubated in a stoppered 250 ml capacity conical flask on a rotary shaker at 100 rpm, 25°C for 48 hours. The beads were collected by filtration on a Buchner funnel, washed with 200 ml of distilled water and dried at 40°C under vacuum. The derivatised macroporous beaded crosslinked copolymer beads were stored at 4°C.

Example 11

10 g of the macroporous beaded crosslinked copolymer prepared as described in Example 4 was suspended in 60 ml of ligand solution containing 28.5 g of phenylethylamine dissolved in isopropanol. The suspension was incubated in a stoppered 250 mi capacity conical flask on a rotary shaker at 100 rpm, 25°C for 48 hours. The beads were collected by filtration on a Buchner funnel, washed with 200 ml of distilled water and dried at 40°C under vacuum. The derivatised macroporous beaded crosslinked copolymer beads were stored at 4°C.

Example 12

10 g of the macroporous beaded crosslinked copolymer prepared as described in Example 4 was suspended in 60 ml of ligand solution containing 25.2 g of benzylamine dissolved in ethanol. The suspension was incubated in a stoppered 250 ml capacity conical flask on a rotary shaker at 100 rpm, 25°C for 48 hours. The beads were collected by filtration on a Buchner funnel, washed with 200 ml of distilled water and dried at 40°C under vacuum. The derivatised macroporous beaded crosslinked copolymer beads were stored at 4°C.

Example 13

10 g of the macroporous beaded crosslinked copolymer prepared as described in Example 1 was suspended in 60 ml of ligand solution containing 25.2 g of benzylamine dissolved in ethanol. The suspension was incubated in a stoppered 250 ml capacity conical flask on a rotary shaker at 100 rpm, 25°C for 48 hours. The beads were collected by filtration on a Buchner funnel, washed with 200 ml of distilled water and dried at 40°C under vacuum. The derivatised macroporous beaded crosslinked copolymer beads were stored at 4°C.

Example 14

10 g of the macroporous beaded crosslinked copolymer prepared as described in Example 3 was suspended in 60 ml of ligand solution containing 25.2 g of benzylamine dissolved in ethanol. The suspension was incubated in a stoppered 250 ml capacity conical flask on a rotary shaker at 100 rpm, 25°C for 48 hours. The beads were collected by filtration on a Buchner funnel, washed with 200 ml of distilled water and dried at 40°C under vacuum. The derivatised macroporous beaded crosslinked copolymer beads were stored at 4°C.

Example 15

10 g of the macroporous beaded crosslinked copolymer prepared as described in Example 6 was suspended in 60 ml of ligand solution containing 25.2 g of benzylamine dissolved in ethanol. The suspension was incubated in a stoppered 250 ml capacity conical flask on a rotary shaker at 100 rpm, 25°C for 48 hours. The beads were collected by filtration on a Buchner funnel, washed with 200 ml of distilled water and dried at 40°C under vacuum. The derivatised macroporous beaded crosslinked copolymer beads were stored at 4°C.

Examples for the purification of penicillin G acylase by using derivatized macroporous beaded crosslinked copolymers

Example 16

20 mls of crude enzyme extract of penicillin G acylase from Escherichia coli prepared in phosphate buffer having molarity of 0.05 and pH 7.5 containing 100 IU of pencillin G acylase was taken in 100 ml capacity container. 1.5 gram of derivatized macroporous beaded crosslinked allyl

glycidyl ether copolymer prepared as described in example 7 was added to the enzyme solution and the suspension was agitated at 100 rpm at 25°C for a period of 30 minutes. The beads were separated by filtration and washed with 30 mls of phosphate buffer having a molarity of 0.05 and pH 7.5, The penicillin G acylase adsorbed onto the beads was eluted by washing in 5 lots of 5 mls of phosphate buffer having molarity of 0.05, pH 7.5 containing phenylacetic acid to a concentration of 0.5 M. The fractions containing penicillin G acylase activity were pooled and subjected to dialysis against phosphate buffer having molarity of 0.05 M and pH of 7.5. The adsorption and recovery of penicillin G acylase was 10 and 5 %, respectively.

Example17

20 mls of crude enzyme extract of penicillin G acylase from Escherichia coli prepared in tris chloride buffer having molarity of 0.07 and pH 7.8 containing 100 IU of pencillin G acylase was taken in 100 ml capacity container. 1.5 gram of derivatized macroporous beaded crosslinked allyl glycidyl ether copolymer prepared as described in example 8 was added to the enzyme solution and the suspension was agitated at 120 rpm at 25°C for a period of 30 minutes. The beads were separated by filtration and washed with 30 mls of tris chloride buffer having a molarity of 0.07 and pH 7.8. The penicillin G acylase adsorbed onto the beads was eluted by washing in 5 lots of 5 mls of tris chloride buffer having molarity of 0.07, pH 7.8 containing phenylacetic acid to a concentration of 0.5 M. Fractions containing penicillin G acylase activity were pooled and dialysed against tris chloride buffer having molarity of 0.07 M and pH of 7.8. The adsorption and recovery of penicillin G acylase was 76 and 67.6 %, respectively.

Example 18

20 mls of crude enzyme extract of penicillin G acylase from Bacillus megaterium prepared in phosphate buffer having molarity of 0.10 and pH 7.0 containing 100 IU of pencillin G acylase was taken in 100 ml capacity container. 1.5 gram of derivatized macroporous beaded crosslinked allyl glycidyl ether copolymer prepared as described in example 9 was added to the enzyme solution and the suspension was agitated at 200 rpm at 30°C for a period of 25 minutes. The beads were separated by filtration and washed with 30 mls of phosphate buffer having a molarity of 0.010 and pH 7.0. The penicillin G acylase adsorbed onto the beads was eluted by washing in 5 lots of 5 mls of phosphate buffer having molarity of 0.10, pH 7.0 containing phenylacetic acid to a concentration of 0.5 M. The fractions containing penicillin G acylase activity were pooled and dialysed against phosphate buffer having molarity of 0.10 M and pH of 7.0. The adsorption and recovery of penicillin G acylase was 100 and 84.2 %, respectively.

Example 19

20 mls of crude enzyme extract of penicillin G acylase from Escherichia coli prepared in phosphate buffer having molarity of 0.05 and pH 7.5 containing 100 IU of pencillin G acylase was taken in 100 ml capacity container. 1.5 gram of derivatized macroporous beaded crosslinked allyl glycidyl ether copolymer prepared as described in example 10 was added to the enzyme solution and the suspension was agitated at 300 rpm at 25°C for a period of 25 minutes. The beads were separated by filtration and washed with 30 mls of phosphate buffer having a molarity of 0.05 and pH 7.5. The penicillin G acylase adsorbed onto the beads was eluted by washing in 5 lots of 5 mls of phosphate buffer having molarity of 0.05, pH 7.5 containing ethylene glycol to a concentration of 0.5 M. The fractions containing penicillin G acylase activity were pooled and dialysed against

phosphate buffer having molarity of 0.05 M and pH of 7.5. The adsorption and recovery of penicillin G acylase was 100 and 82.5 %, respectively.

Example 20

20 mls of crude enzyme extract of penicillin G acylase from Escherichia coli prepared in tris chloride buffer having molarity of 0.20 and pH 8.0 containing 100 IU of pencillin G acylase was taken in 100 ml capacity container. 1.5 gram of derivatized macroporous beaded crosslinked allyl giycidyl ether copolymer prepared as described in example 11 was added to the enzyme solution and the suspension was agitated at 100 rpm at 25°C for a period of 30 minutes. The beads were separated by filtration and washed with 30 mls of tris chloride buffer having a molarity of 0.20 and pH 8.0. The penicillin G acylase adsorbed onto the beads was eluted by washing in 5 lots of 5 mls of tris chloride buffer having molarity of 0.20, pH 8.0 containing phenylacetic acid to a concentration of 0.5 M. The fractions containing penicillin G acylase activity were pooled and dialysed against tris chloride buffer having molarity of 0.20 M and pH of 8.0. The adsorption and recovery of penicillin G acylase was 97 and 85.5 %, respectively.

Example 21

20 mls of crude enzyme extract of penicillin G acylase from Escherichia coli prepared in tis chloride buffer having molarity of 0.15 and pH 7.5 containing 100 IU of pencillin G acylase was taken in 100 ml capacity container. 1.5 grams of derivatized macroporous beaded crosslinked allyl giycidyl ether copolymer prepared as described in example 12 was added to the enzyme solution and the suspension was agitated at 100 rpm at 25°C for a period of 30 minutes. The beads were separated by filtration and washed with 30 mls of tris chloride buffer having a molarity of 0.15 and

pH 7.5. The penicillin G acylase adsorbed onto the beads was eluted by washing in 5 lots of 5 mls of tris chloride buffer having molarity of 0.15, pH 7.5 containing propylene glycol to a concentration of 0.5 M. The fractions containing penicillin G acylase activity were pooled and dialysed against tris chloride buffer having molarity of 0.15 M and pH of 7.5. The adsorption and recovery of penicillin G acylase was 90 and 82.3 %, respectively. The specific activity of the purified penicillin G acylase was 12.2 IU per mg protein. Thus, 4.9-fold purification was achieved.

Example 22

20 mls of crude enzyme extract of penicillin G acylase from Escherichia coli prepared in phosphate buffer having molarity of 0.05 and pH 7.5 containing 100 IU of penicillin G acylase was taken in 100 ml capacity container. 1.5 gram of derivatized macroporous beaded crosslinked allyl glycidyl ether copolymer prepared as described in example 15 was added to the enzyme solution and the suspension was agitated at 100 rpm at 25°C for a period of 30 minutes. The beads were separated by filtration and washed with 30 mls of phosphate buffer having a molarity of 0.05 and pH 7.5. The penicillin G acylase adsorbed onto the beads was eluted by washing in 5 lots of 5 mls of phosphate buffer having molarity of 0.05, pH 7.5 containing phenylacetic acid to a concentration of 0.5 M. The fractions containing penicillin G acylase activity were pooled and dialysed against phosphate buffer having molarity of 0.05 M and pH of 7.5. The adsorption and recovery of penicillin G acylase was 100 and 87 %, respectively. The specific activity of the purified penicillin G acylase was 14 IU per mg protein.

Advantages of the invention

The invention provides a process for the preparation of purified penicillin G acylase from an enzyme extract of *Escherichia coli* cells and offers the following advantages.

1. High recovery up to 87 % of penicillin G acylase can be obtained.
2. The operations involve single step of affinity interaction chromatography.
3. The process developed is a batch operation and use of column chromatography equipments is not required.
4. The purified penicillin G acylase obtained is useful for immobilization onto other solid matrices.