Purification and characterization of an anti-cancer enzyme produced by marine *Vibrio Costicola* under a novel solid state fermentation process

G. Nagendra Prabhu*; M. Chandrasekaran

Microbial Technology Unit, Department of Biotechnology, Cochin University of Science & Technology, Cochin - 682 022, India
*Present address: P. G. Department of Zoology & Research Centre, S.D. College, University of Kerala, Alleppey - 688 003, India

MATERIALS AND METHODS

Micro-organism and solid state fermentation: *Vibrio costicola* ACMR 267 isolated from the marine environment of Cochin, Kerala, India, and maintained on ZoBell's marine agar slants, was used (Renu & Chandrasekaran, 1992). Enzyme production under SSF using polystyrene beads was carried out according to a new protocol developed by the authors. 5 gm of polystyrene beads, taken in 250 ml Erlenmeyer flasks were moistened with 5 ml of aged sea water media containing L-glutamine (2% w/w), maltose (1% w/w) and $KH_2 PO_4$ (1% w/w). After sterilisation, the flasks were inoculated with 2 ml of inoculum (0.750 mg dry wt /10 gm substrate) and incubated at 35^0C for 24 h under 75-80% relative humidity. L-Glutaminase was extracted using phosphate buffer (0.1M, pH 7.0, 1:5 w/v per flask). After extracting twice, the extracts were pooled, centrifuged at 10000 rpm for 30 min at 4ºC and the supernatant was used as the crude enzyme (Nagendra Prabhu & Chandrasekaran, 1995, 1997).

Enzyme purification: L-Glutaminase was purified by ammonium sulphate fractionation followed by dialysis and gel-filtration chromatography. Ammonium sulphate fraction-ation was carried out at 4ºC in an ice bath. The precipitated protein was collected by centrifugation at 10000 rpm at 4ºC and dissolved in a minimum volume of phosphate buffer (0.01M, pH 8.0). Fractions obtained in the range of 50 - 80% saturation of ammonium sulphate were pooled and dialysed against the same buffer for 24 h at 4ºC with continuous stirring and occasional changes of the buffer. The dialysate was concentrated by lyophilization and applied on top of a gel-filtration column (1.5 x 50-cm) containing Sephadex G 100. Elution was carried out in a cold room using phosphate buffer (0.1M, pH 8.0) containing 0.1M NaCl and 0.001M EDTA. Fractions of 2.5 ml were collected at a flow rate of 50 ml/h. Each fraction was analysed for protein and glutaminase. Active fractions were pooled and concentrated by lyophilization (Nagendra Prabhu, 1997). Purity was confirmed by polyacrylamide gel electrophoresis under denaturing and non-denaturing conditions (Hames, 1990)

Analytical techniques: Protein was estimated by Lowry's method (Lowry *et al*, 1951) or by measuring absorbance at 280 nm. L-Glutaminase was assayed according to Imada *et al* (1971). One international unit of glutaminase was defined as the amount of enzyme that liberates 1 µmol of ammonia under optimal assay conditions. Enzyme yield was expressed as Units/gm dry substrate (U/gds). Viscosity of the leachate (extractant) was measured using Ostwald viscometer and expressed as Newtons/m^2 (Ns/m^2).

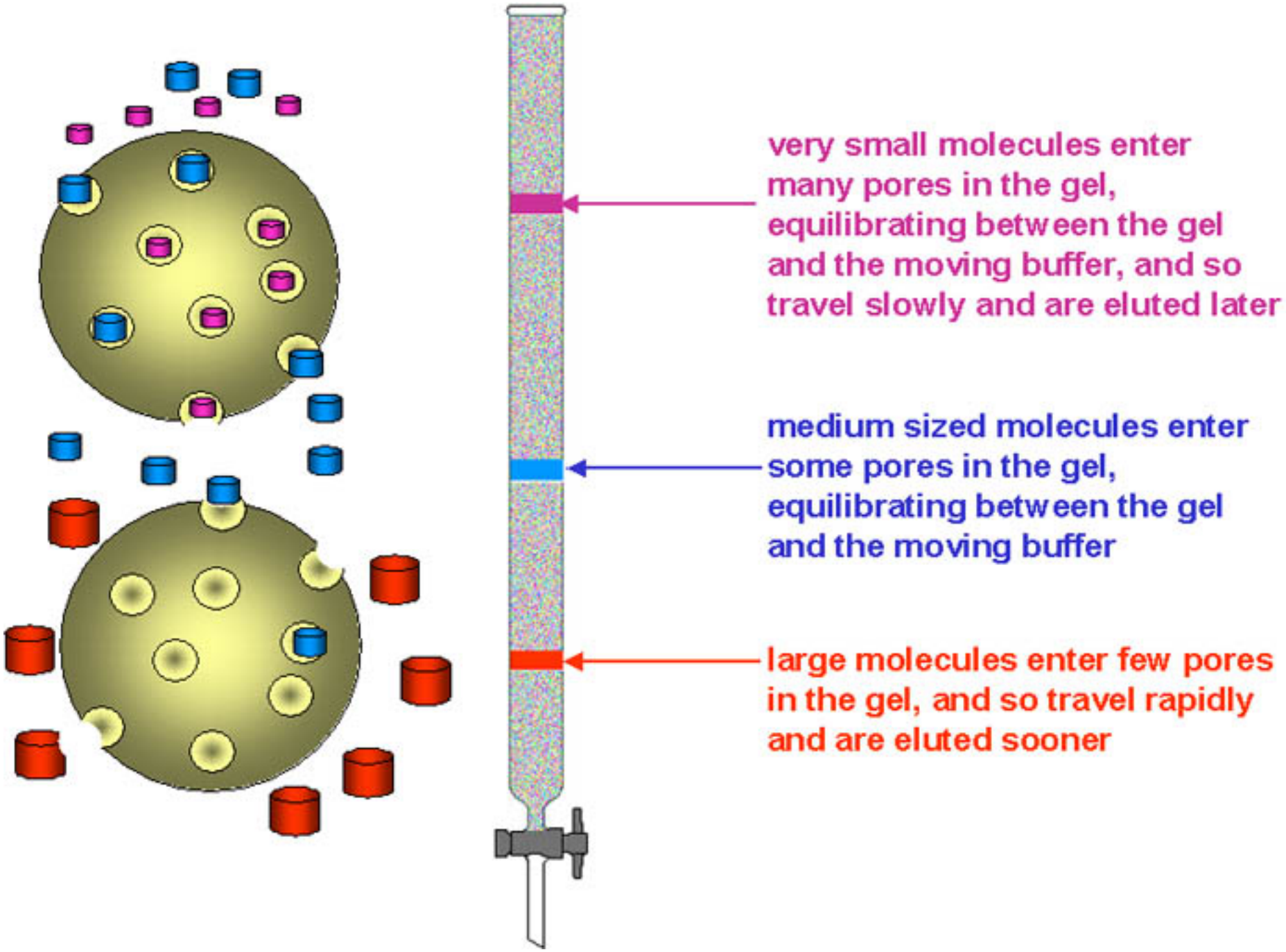
Enzyme characteristics: The purified glutaminase was characterised for its various properties. The characters analysed included effect of pH (4 - 10) and temperature (30 - 80ºC) on enzyme activity and stability, effect of NaCl (0 - 20%) on enzyme activity, effect of substrate concentration (L - glutamine; 0.01 - 1.0 M) on enzyme activity, effect of L-glutamine (0.01M) and NaCl (10%) on temperature stability of glutaminase and the substrate specificity the enzyme (Nagendra Prabhu, 1997).

G-10 is well suited for the separation of biomolecules such as peptides (MW>700) from smaller molecules (MW <100).
G-50 is suitable for the separation of molecules MW >30 000 from molecules MW <1 500 such as labeled protein or DNA from unconjugated dyes. The medium is often used to remove small nucleotides from longer chain nucleic acids.
G-25 is recommended for the majority of group separations involving globular proteins. These media are excellent for removing salt and other small contaminants away from molecules that are greater than MW 5000. Using different particle sizes enables columns to be packed according to application requirements, see Table 5.1. The particle size determines the flow rates and the maximum sample volumes that can be applied. For example, smaller
particles give higher column efficiency (narrow, symmetrical peaks), but may need to be run more slowly as they create higher operating pressures.
G-100 is recommended for molecular weight determination

Applications

Desalting columns are used not only to remove low molecular weight contaminants such as salt, but also for buffer exchange before and after different chromatography techniques and for the rapid removal of reagents to terminate a reaction. Examples of group separations include:

- Removal of phenol red from culture fluids prior to anion exchange chromatography or nucleic acid preparations
- Removal of unincorporated nucleotides during DNA sequencing
- Removal of free low molecular weight labels
- Termination of reactions between macromolecules and low molecular weight reactants
- Removal of products, cofactors or inhibitors from enzymes
- Removal of unreacted radiolabels such as [_-32P] ATP from nucleic acid labeling reactions


very small molecules enter many pores in the gel, equilibrating between the gel and the moving buffer, and so travel slowly and are eluted later
medium sized molecules enter some pores in the gel, equilibrating between the gel and the moving buffer
large molecules enter few pores in the gel, and so travel rapidly and are eluted sooner

ABSTRACT

L - Glutaminase, a therapeutically and industrially important enzyme, was produced from marine Vibrio costicola by a novel solid state fermentation process using polystyrene beads as inert support. The new fermentation system offered several advantages over the conventional systems, such as the yield of leachate with minimum viscosity and high specific activity for the target product besides facilitating the easy estimation of biomass. The enzyme thus produced was purified and characterised. It was active at physiological pH, showed high substrate specificity towards L - glutamine and had a Km value of 7.4×10^{-2} M. It also exhibited high salt and temperature tolerance indicating good scope for its industrial and therapeutic applications.

Key words: L- Glutaminase, *Vibrio costicola*, solid state fermentation, inert support, purification and characterisation

Enzyme Research
Volume 2014, Article ID 120739, 6 pages
http://dx.doi.org/10.1155/2014/120739

Research Article

Purification and Characterization of Melanogenic Enzyme Tyrosinase from Button Mushroom

Introduction

Tyrosinase (E.C. 1.14.18.1) is a ubiquitous enzyme involved in pigmentation. It catalyzes hydroxylation of monophenols (cresolase activity) and oxidation of diphenols (catecholase activity) in the presence of molecular oxygen. The conversion of phenols to *o*-diphenols by tyrosinase is a potentially attractive catalytic ability and thus tyrosinase has attracted a lot of attention with respect to its biotechnological application as the catechol products are useful as drugs or drug synthons, for example, L-DOPA [1]. It also plays an important role in the formation of melanin pigment during melanogenesis in melanocytes which are located at the epidermal junction and it is present in these cells that originate from the embryonic neural crest and is responsible for the synthesis of melanin. [2] It was first characterized in mammals for its role in the development of melanomas and for implication in pigmentation troubles such as albinism and vitiligo [3]. The physiological role of tyrosinases is related to melanin biosynthesis and has been extracted from different sources such as fungi, fruits, and mammalian melanoma tumors [4, 5]

2.1. Preparation of Tyrosinase

Extraction of mushroom tyrosinase was performed by the method of Kamahldin et al. [16], with few modifications. The sliced mushrooms were homogenized by waring blender. Enzyme extraction was prepared with 500 mL of cold 100 mM phosphate buffer (pH 5.8) for 300 g of mushroom. The homogenate was centrifuged at 5000 rpm for 30 min and supernatant was collected. The sediments were mixed with cold phosphate buffer and were allowed to stand in cold condition with occasional shaking. Then the sediment containing buffer was subjected to centrifugation once again to collect supernatant. The supernatant was used as a source of enzyme.

2.2. Purification of the Enzyme from the Crude Extract

The purification of tyrosinase was performed by the method of Kamahldin et al. [16], with minor modification. Crude enzyme extract purified by salt precipitation, dialysis, gel filtration, ion exchange chromatography, and so forth has been employed in series so as to obtain the enzyme in its purest form. The pure enzyme thus produced can be used for the further analysis.

2.3. Ammonium Sulphate Precipitation and Dialysis

Ammonium sulphate precipitation was done in an ice bath using the finely grounded ammonium sulfate. The powder was weighed and added slowly to the extract by constant stirring to ensure complete solubility, and the solution was centrifuged at 5000 rpm for 30 min at 4°C. Different precipitation steps were carried out for tyrosinase enzyme precipitation (45–80%) and precipitates were collected. The precipitate was dialyzed against 100 mM potassium phosphate buffer (pH 7.0) for 24 h by changing the buffer thrice. The dialyzed fraction was used for tyrosinase activity and protein content

2.4. Assay of Tyrosinase Activity

The tyrosinase activity assay was performed as reported by Sung and Cho [17] spectrophotometrically, measuring conversion of L-DOPA to red colored oxidation product dopachrome. The initial rate of reaction is proportional to concentration of the enzyme. An aliquot containing tyrosinase was incubated for 5 min at 35°C at time zero, 1 mL of L-DOPA solution (4 mg/mL) for measured at 475 nm. After incubation for additional 5 min, the mixture was shaken again and a second reading was determined and was measured for 3 minutes. The change in absorbance was proportional to enzyme concentration. One unit of enzyme corresponded to the amount which catalyzed the transformation of 1 μmol of substrate to product per min under the above conditions and produced 1.35 changes in absorbance. Specific activity was expressed as enzyme unit per milligram of protein. The protein content of the enzyme was determined by the method of Lowry [18], with bovine serum albumin as standard.

2.5. Sephadex G-100 Gel Filtration

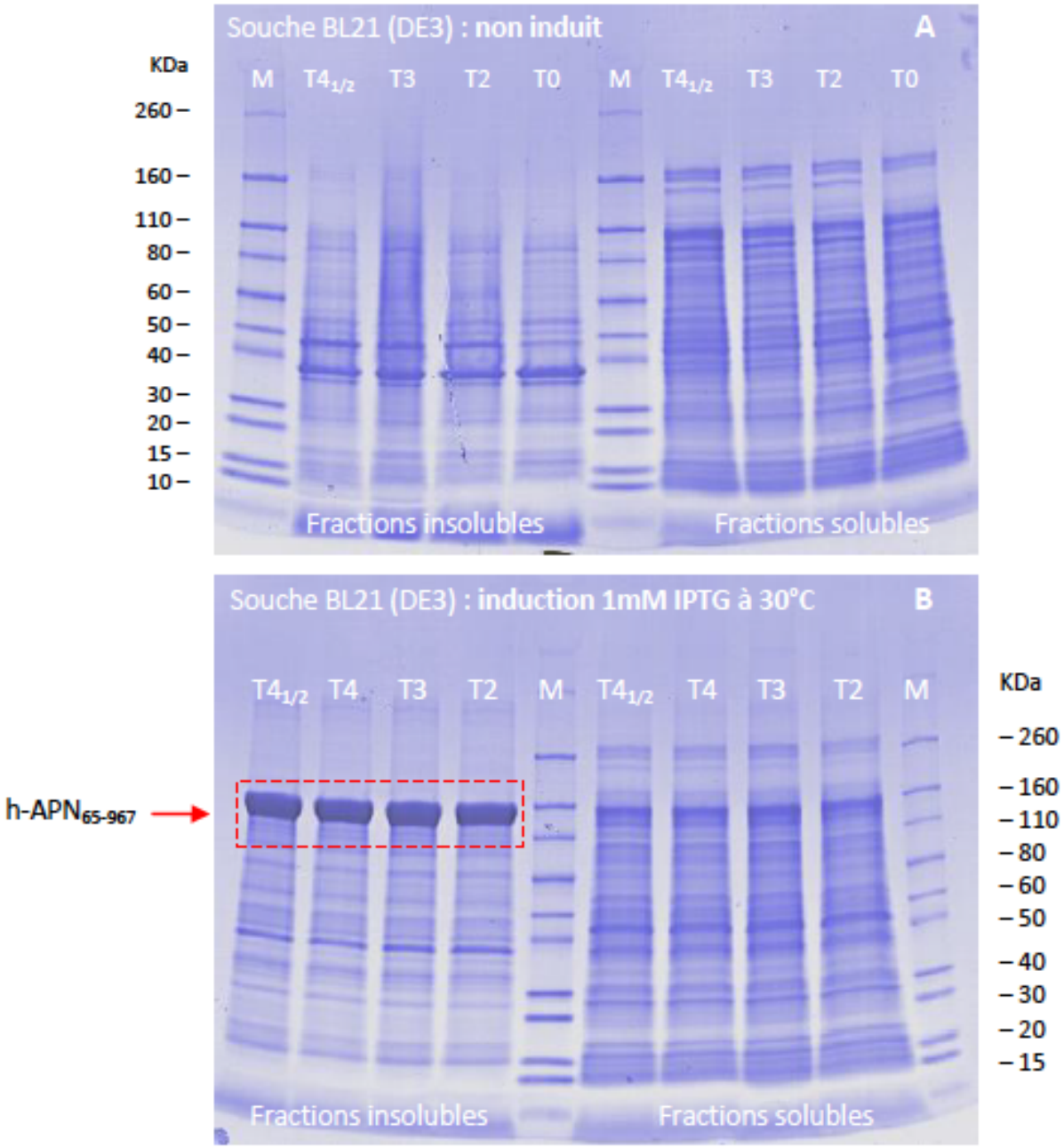
The dialyzed ammonium sulfate fraction was applied to a Sephadex G-100 column that was preequilibrated with a 100 mM phosphate buffer of pH 7.0. The protein elution was done with the same buffer at a flow rate of 5 mL/min. The fractions were collected at 4°C. It was assayed for protein at 280 nm as well as for enzyme activity. The active fractions were pooled, dialyzed against the 100 mM phosphate buffer of pH 7.0, and concentrated.

2.6. DEAE-Cellulose Column Chromatography

Dialyzed enzyme preparation obtained after ammonium sulphate precipitation and Sephadex G-100 column was subjected to ion exchange chromatography using DEAE-Cellulose column (20 × 1 cm). The dialyzed enzyme preparation was loaded on DEAE-Cellulose column which was preequilibrated with potassium phosphate buffer (100 mM, pH 7.0). The column was washed first with equilibrated buffer and then bound proteins were eluted using linear gradient of 0–100 mM NaCl and 0–100 mM potassium phosphate buffer at a flow rate of 1 mL per min. The fractions (2.5 mL each) were collected and assayed for tyrosinase activity and those showing high activity were pooled and used for SDS-PAGE analysis

2.7. Sodium Dodecyl Sulfate-Polyacrylamide Gel (SDS-PAGE) Electrophoresis of Purified Tyrosinase

SDS-PAGE was performed using a 12% separating gel and 4% stacking gel. The samples were heated for 5 min at 100°C in capped vials with 1% (w/v) SDS in the presence of *β*-mercaptoethanol. Electrophoresis was performed at a 125 V for 4 h in Tris-HCl buffer of pH 8.3. After electrophoresis, proteins in the separating gel were made visible by staining with Coomassie Brilliant Blue R-250. The standards used to make a plot of log molecular weight versus mobility of the protein band were lysozyme (20 kDa), myoglobin (26 kDa), carbonic anhydrase (38 kDa), ovalbumin (46 kDa), glutamate (62 kDa), bovine serum albumin (91 kDa), *β*-galactosidase (120 kDa), and myosin (200 kDa).


Souche BL21 (DE3) : non induit
A
KDa
M
T4 1/2
T3
T2
T0
260
160
110
80
60
50
40
30
20
15
10
Fractions insolubles
Fractions solubles
Souche BL21 (DE3) : induction 1mM IPTG à 30°C
B
T4 1/2
T4
T3
T2
M
h-APN65-967
Fractions insolubles
Fractions solubles