An overview of purification methods of glycoside hydrolase family 70 dextranase

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Abstract The enzyme dextranase (sucrose:1, 6- α -D-glucan 6- α -glucosyltransferase, EC 2.4.1.5) catalyses the synthesis of exopolysaccharide, dextran from sucrose. This class of polysaccharide has been extensively exploited in pharmaceutical industry as blood volume expander, as stabiliser in food industry and as a chromatographic medium in fine chemical industry because of their non-ionic nature and stability. Majority of the dextrans are synthesized from sucrose by dextranase secreted mainly by bacteria belonging to genera *Leuconostoc*, *Streptococcus* and *Lactobacillus*. Bulk of the information on purification of extracellular dextranase has been generated from *Leuconostoc* species. Various methods such as precipitation by ammonium sulphate, ethanol or polyethylene glycol, phase partitioning, ultrafiltration and chromatography have been used to purify the enzyme. Purification of dextranase is rendered difficult by the presence of viscous dextran in the medium. However, processes like

ultra-filtration, salt and PEG precipitation, chromatography and phase partitioning have been standardized and successfully used for higher scale purification of the enzyme. A recombinant dextranase from *Leuconostoc mesenteroides* B-512F with a histidine tag has been expressed in *E. coli* cells and purified by immobilized metal ion chromatography. This review reports the available information on purification methods of dextranase from *Leuconostoc mesenteroides* strains.

Keywords *Leuconostoc mesenteroides* · glucosyltransferase · dextranase · dextran · glucan · glycoside hydrolase

Introduction

Dextranase [EC 2.4.1.5] has been included in glycoside hydrolases based on their sequence homologies. Glycoside hydrolases have been grouped till date into 108 families (<http://afmb.cnrs-mrs.fr/CAZY/>). The enzyme dextranase belongs to family 70 of glycoside hydrolases. The dextranase consists of N-terminal two-third catalytic domain and C-terminal glucan-binding domain containing a series of direct repeating units¹. Dextranase is produced by microorganisms belonging to families Lactobacillaceae and Streptococcaceae, especially by the genera *Lactobacillus*, *Leuconostoc* and *Streptococcus*, with *L. mesenteroides* being the most extensively studied for dextran and dextranase production. The dextranases of *Streptococcus* species are produced constitutively but those from *Leuconostoc mesenteroides* are produced by sucrose induction², except for its recently isolated constitutive mutants^{3,4}. The soluble dextran produced by

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dextranase has gained importance owing to its several commercial applications in clinical, pharmaceutical, food, photo film, fine chemical and other industries. Dextranase of *Leuconostoc* spp. is induced by sucrose, which leads to concomitant dextran synthesis during enzyme production. The presence of dextran results in aggregated forms of enzyme. In addition, dextrans make the culture supernatants viscous, making enzyme purification cumbersome. Various methods like ultra-filtration, salt and PEG precipitation, chromatography and phase-partitioning, and their combinations used for purification, are reviewed in the following text. The first attempt to purify dextranase involved ammonium sulfate precipitation⁵. A series of reports appeared on various methods of purification since then. A wide variety of techniques have been used for the purification of *Leuconostoc mesenteroides* dextranase^{6–15}. A recent report describes the production of recombinant dextranase of *L. mesenteroides* B-512F with a histidine tag at the C-terminal using *E. coli* TOP10 cells for expression and purification of the recombinant enzyme by affinity chromatography using Ni²⁺ ion immobilized column¹⁶.

Dextranase production

The dextranase production has been thoroughly reviewed and documented in the reviews^{17,18}. Various *Leuconostoc mesenteroides* strains and methods have been used for production of dextranase^{6,10,19–22}. Successful attempts have been made to improvise the strain or enzyme production. The maintenance and production media composition and culture conditions have been optimized for the large scale production of dextranase. The low cost carbon and nitrogen sources like sugar-beet molasses, corn steep liquor and wheat bran extract have been successfully employed for large-scale preparation of dextranase by fermentation process. Mutants of *Leuconostoc mesenteroides* strains have been developed³, and fermentation techniques like batch, semi-continuous fermentation by free and immobilized cells were tried to economize commercial production of dextranase.

Structural characteristics of dextranase

Several workers have reported that dextranase exists in single or multiple forms having molecular weight in the range 64,000 – 245,000^{8,11,12,14,15,23}. The enzyme remains in an aggregated form in the presence of dextran resulting in high molecular weight. The aggregated forms disassociate into low molecular weight forms by endodextranase treatment⁸. The purified enzyme is stable

and active only in the presence of dextran and in the absence of dextran dextranase loses activity and this loss could be prevented by adding dextran²⁴. It was also reported that dilute enzyme lost activity rapidly and the addition of dextran prevented the loss of activity. Kinetic studies of the dextranase are affected by the presence of dextran. The enzyme can be stabilized by high-molecular-weight dextran, polyethyleneglycol or nonionic detergents such as Tween 80^{14,24}. The enzyme exhibited a K_m of 2 mM for sucrose and an apparent optimum temperature of 30°C. Dextranase is inactivated by EDTA and can be reactivated with Ca²⁺. Dextranase is strongly inhibited by metal ions such as Cu²⁺, Fe³⁺ and Mn²⁺²⁵. Diethyl pyrocarbonate (DEP) was shown to inhibit the enzyme activity, which indicated the presence of essential histidine residues at the active site²⁶. Statistical and kinetic analyses of the inactivation of enzyme by DEP showed that two histidine residues are essential for the enzymatic activity. A lysine residue at the active site was shown to play an important role in the catalytic activity of dextranase²⁷. The presence of an essential lysine residue at the active site was shown by 2,4,6-trinitrobenzene-sulphonic acid²⁷, pyridoxal-5'-phosphate²⁸ and *o*-phthalaldehyde²⁹ inactivation studies. And a single but non-essential cysteine residue was located near the active site of dextranase by chemical modification studies and amino acid analysis³⁰. Structural-functional relationships of glucanases from lactic acid bacteria have been extensively documented in the reviews^{31,32}.

Dextrans

Dextrans are polymeric chain of glucosyl units, synthesized by dextranase by the transfer of D-glucosyl unit from sucrose to acceptor molecules. This polymerisation results in formation of dextran and fructosyl unit is released free. Dextrans also called glucans, feature substantial number of consecutive $\alpha(1,6)$ -linkages in their main chain, usually comprising more than 50% of the total linkages. These α -D-glucans also possess side chains, stemming mainly from $\alpha(1,3)$ - and occasionally from $\alpha(1,4)$ - or $\alpha(1,2)$ -branched linkages. A survey of ninety-six strains of glucan producing bacteria was done to classify dextrans by their structure and properties and identify a suitable strain, for pharmaceutical industry³³. Different types of dextrans of varying size and structure are synthesized depending on the dextranase produced by the strain^{34–37}. Dextrans are classified into three classes A, B and C on the basis of the percentage of 1,3-like linked anhydroglucopyranose units (AGU) as determined by periodate oxidation³⁸.

Applications of Dextran

Soluble dextrans have well documented wide applications in industry^{16–18,39}. The dextranucrase activity of *L. mesenteroides* was used in acceptor reactions with maltose in order to obtain oligosaccharides with α -1,2 glycosidic bonds^{19,40}. These compounds resist hydrolysis by the digestive enzymes in animals and humans because of the configuration of their glycosidic bonds and selectively stimulate intestinal denizens like *Bifidobacterium* spp. *Lactobacillus* spp. and *Bacteroides* spp.⁴¹. Thus, dextrans act as prebiotics, promising a benefit to human health. They are already being used as additives in animal and human nutrition^{17,41–43}. Dextrans are widely used in pharmaceutical industry as blood volume expander and in food industry as conditioning, stabilising and bodying agent replacing natural gums⁴⁴. It is used against iron deficiency anemia and in open wound healing⁶. The derivatives of dextran like sephadex and DEAE-dextran serve as molecular sieves and are extensively used for separation of biomolecules in chromatography^{35,45,46}.

Purification of dextranucrase

Precipitation by salt and solvent

In 1946, E.J. Hehre obtained crude preparations of dextranucrase from *L. mesenteroides* by ammonium sulfate precipitation. Ammonium sulfate was added to the bacterial culture and the pellet obtained after centrifugation containing the cells and enzyme was further freed from preformed polysaccharides by washing with saturated ammonium sulfate⁵. The supernatant obtained after centrifugation was treated with an equal volume of acetate buffer and centrifuged at low temperature. The precipitate obtained was used as the enzyme preparation⁵. Koepsell and Tsuchiya used both, alcohol and ammonium sulfate precipitation to obtain purified dextranucrase as a lyophilized dry powder with 100 U mg^{-1} specific activity from *L. mesenteroides* B-512. The culture supernatant of *L. mesenteroides* NRRL B-1299 was subjected to ammonium sulfate fractionation. The enzyme was collected, dialyzed and used as enzyme preparation that had a specific activity of 0.1 U mg^{-1} . The recovery of protein by this method was very low⁴⁷. The cells from culture of *L. mesenteroides* LM₁ were centrifuged and the pellet was washed to remove any preformed dextran and was resuspended in acetate buffer, followed by the addition of sucrose and toluene⁴⁸. The addition of sucrose induced dextranucrase secretion into the suspension. The suspension was centrifuged and the secreted enzyme was

precipitated by ammonium sulfate. An enzyme activity of 2.96 U mg^{-1} was obtained that was not significant⁴⁸. Robyt and Walseth found the ammonium sulfate to be completely ineffective in precipitating dextranucrase from a cell free culture supernatant as more than 90% of the dextranucrase activity was lost when the concentration of ammonium sulfate was 80% (w/v)⁶. Purification with ammonium sulfate thus led to considerable loss of enzyme. In general ammonium sulfate did not prove to be an agent that could efficiently precipitate the dextranucrase.

Alcohol has also been used to precipitate dextranucrase. Pre-chilled ethanol was added to the cell free supernatant of *L. mesenteroides* B-512 with an activity of 40–50 DSU, for precipitation of enzyme⁴⁹. The precipitate was collected by centrifugation and the resulting enzyme gave 700 DSU, where one DSU was defined as the amount of enzyme required to convert 1 mg of sucrose to dextran in 1 h at 30°C and pH 5.0, as determined by increase in concentration of fructose⁴⁹. The precipitation of enzyme with alcohol removed a significant amount of dextran with it. Glycerol has also been used to precipitate the enzyme dextranucrase. The addition of 50% glycerol to the culture supernatant obtained after centrifugation resulted in dextranucrase with an activity of 64 U ml^{-1} where, one unit of activity was defined as the amount of enzyme in micromole/ml that converts 1 mg of sucrose into dextran in 1 h at 30°C and pH 5.0⁵⁰. The major drawback of this method was that the purified enzyme contained dextran.

Fractionation by polyethylene glycol

Polyethylene glycol (PEG) is known to selectively precipitate proteins, which have high molecular weights or exist in aggregated forms. The large size of dextranucrase, together with its tendency to form aggregates in solution has led to the use of non-ionic hydrophilic polymer PEG for precipitation of dextranucrase. Dextranucrase from *Streptococcus mutans* was purified by PEG precipitation using PEG 400 and 6000⁵¹. PEG 6000 precipitated other non-dextranucrase proteins, while PEG 400 gave higher specificity of precipitation and had an advantage of being readily removed by dialysis⁵¹. A simple and effective method of purification of dextranucrase from *L. mesenteroides* NRRL B-512F, using PEG precipitation was developed¹⁴. The crude dextranucrase from culture supernatant with an activity of 1.4 U ml^{-1} was subjected to PEG of different molecular weights. The PEG 400 reproducibly gave dextranucrase with specific activity of 8.7 U mg^{-1} , with 80% yield and 15-fold purification. In a typical experiment, in successive precipitation steps of fractionation performed on the crude

enzyme using PEG 400, the specific activity increased from 0.58 to 29 Umg⁻¹, resulting in 50 fold purification with 70% overall yield after the three steps. In the subsequent fourth step, the activity decreased sharply at all the concentrations of PEG 400¹⁴. This was probably due to the low levels of dextran left in the enzyme preparation as a consequence of dextran removal by PEG. The removal of dextran, might have caused a decrease in activity for dextransucrase, being a stabilizer for dextransucrase. High molecular weight PEG 20,000 has also been used to fractionate the enzyme from *L. mesenteroides* B-512FM⁵². The final enzyme obtained after dialysis had an activity of 89 Umg⁻¹. PEG precipitation is relatively the easier and faster procedure to obtain purified form of dextransucrase. The enzyme preparations are also devoid of contaminating enzymes like levansucrase and invertase. The major disadvantage of this procedure is the contamination by the polysaccharide. Dextran most often remains as a contaminant. PEG is also present in the purified fractions but can be completely removed by extensive dialysis^{51,52}. Dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 was purified using PEG 1500 and gel-filtration⁵³. The PEG 1500 (10%, w/v) gave dextransucrase with maximum specific activity of 23 Umg⁻¹ with 40 fold purification in a single step. The purified enzyme showed multiple molecular forms on SDS-PAGE, however the same sample showed a single band on non-denaturing native-PAGE. The purified dextransucrase confirmed the presence of dextran, when run on SDS-PAGE under non-denaturing gels for *in-situ* activity detection by Periodic Acid Schiff staining. The activity bands corresponded to the native and active form of the purified dextransucrase of approximately, 180 kDa molecular size, that appeared on the denaturing gels stained with Coomassie Brilliant Blue. Further purification of 10% PEG 1500 purified dextransucrase by gel-filtration gave dextransucrase with specific activity of 35 with 61 fold purification⁵³.

Phase-partitioning

Phase-partitioning method has also been applied to purify extracellular dextransucrase. Phase partitioning occurs between dextran and PEG¹⁰. When sucrose is added to the culture supernatant, the enzyme synthesizes dextran in large amounts. Addition of PEG solution to a dextran-rich aqueous solution, leads to the appearance of two-phases; the top phase which is rich in PEG while the bottom one is rich in dextran. Dextransucrase preferentially goes in to the lower dextran-rich phase because of the covalent association of dextransucrase and dextran. The exocellular dextransucrase was purified by aqueous two-phase partitioning

between the dextran present in the culture supernatant of *L. mesenteroides* B-512 and polyethylene glycol¹⁰. PEG 1500 was added to the supernatant and dextransucrase was obtained in the dextran-rich phase in a highly purified form. Successive phase partitioning steps resulted in an enzyme preparation with specific activity of 175 Umg⁻¹ and yield of 95% and the preparation was free of levansucrase activity¹⁰. Otts and Day purified dextransucrase of *L. mesenteroides* ATTC 10830 by two-phase partitioning using PEG 3350⁵⁴. The culture supernatant was added with 10% dextran T-500 solution followed by 20% PEG 3350 addition. The upper phase, consisting of PEG, was removed and to the lower dextran rich phase PEG 3350 solution was added again. This procedure gave 95% recovery of dextransucrase with specific activity of 30 Umg⁻¹⁵⁴. Phase partitioning of PEG and preformed dextran in culture supernatant of *L. mesenteroides* B-742 was carried out with PEG 1500 and an enzyme preparation with an activity of 83 Umg⁻¹ was obtained⁵⁵.

Dextran T70 or soluble native dextran from *L. mesenteroides* NRRL B-1299 was added to culture supernatant followed by PEG 1500 (15% v/v) addition and the dextran rich phase was concentrated by centrifugation⁵⁶. An activity of 4.3 Uml⁻¹ was obtained in the pellet, where one unit corresponds to the amount of enzyme that produces 1 μmol of fructose per minute at 30°C and pH 5.4⁵⁶. Dextransucrase from cultures of *L. mesenteroides* B-512F grown on different carbon sources was recovered by the removal of cells by passing the culture supernatant through a membrane of 0.2 μm⁵⁷. The enzyme was concentrated by aqueous two-phase partitioning with 25% (w/v) PEG 1500. Dextran T70 (1.5%) was added to the supernatant produced from carbon sources other than sucrose, as dextran is only synthesized in the presence of sucrose. Of all the carbon sources used, sucrose showed formation of dextran and with maximum dextransucrase purification, with an activity of 2.4 Umg⁻¹⁵⁷. The purification of dextransucrase was carried out by a four step PEG phase partitioning method using 50% (w/v) PEG 1500 from the culture supernatant of *L. mesenteroides* B-512F⁵⁸. The enzyme preparation had an activity of 62.3 Umg⁻¹, with a carbohydrate content of 233 mgml⁻¹⁵⁸. Affinity phase partitioning using PEG 6000 and 400 was carried out for dextransucrase purification¹⁵. The dextran was generated by adding 5% sucrose to the culture supernatant of *L. mesenteroides* B-512F. Three steps of repeated phase-partitioning by PEG 6000 and PEG 400 showed that the purification of dextransucrase by PEG 6000 was much greater than that obtained by PEG 400. The specific activity of 42.1 Umg⁻¹ and an overall yield of 84% obtained after the third step of phase-partitioning by PEG 6000, were significantly higher than the specific activity of 23.8 Umg⁻¹

and 46% overall yield by PEG 400¹⁵. The procedures for purifying dextranase discussed above either had low yields or failed to remove the associated polysaccharides. For characterization of the enzyme it should be essentially free of the carbohydrate content and the yields of purification should be high. This was achieved by a combination of dextranase treatment, ion-exchange and affinity chromatography after obtaining the crude form of enzyme through ammonium sulfate or PEG precipitation.

Chromatography

Chromatographic separation has been used to obtain polysaccharide free enzyme. Various matrices have been used for the purification of dextranase from the culture supernatants. Dextranase has been purified using columns containing hydroxyapatite, DEAE-cellulose, DEAE-sephadex, sephadex, ultrogel AcA 34. Dextranase binds to dextrans of various sizes. This led to the use of Sephadex gels, which have an affinity to dextran. These gels act as affinity material for the purification of the enzyme from *Leuconostoc* and *Streptococcus* species. Sephadex has been found to be good affinity matrix for dextranase from *Streptococcus*, but poor for *Leuconostoc*^{25,59}. The latter is probably due to the endogenous dextran present in the *Leuconostoc* preparations. The culture supernatant of *L. mesenteroides* B-512 was added with ammonium sulfate to a saturation of 75% after incorporating egg white albumin at a 5% concentration⁶¹. The addition of egg white albumin was effective resulted in a good precipitation of dextranase. The precipitate obtained was subjected to chromatography on DEAE-cellulose column and the enzyme adsorbed on the column was eluted with NaCl gradient. The enzymatically active fractions were collected and reprecipitated with ammonium sulfate. This preparation was applied to DEAE-cellulose column. The active fractions obtained were pooled and then applied to a column of BioGel P-150 and dextranase obtained had a specific activity of 110 U_{mg}⁻¹⁶⁰. The culture supernatant of *L. mesenteroides* B-512 was concentrated, and protein was precipitated with 80% (w/v) ammonium sulfate⁶¹. This preparation was applied to and eluted from hydroxyapatite column with a potassium dihydrogen phosphate gradient and the enzyme gave a specific activity of 3.3 U_{mg}⁻¹. The enzyme when purified using hydroxyapatite was contaminated with levansucrase, invertase and dextranase. Furthermore, the total yield of dextranase was also less than 10%. Therefore, the low yield, the dilution of the dextranase activity and the presence of contaminating enzymes restricted the use of hydroxyapatite⁶¹. The precipitation of enzyme from culture

supernatant of *L. mesenteroides* B-512F was done using ammonium sulfate with egg albumin as co-precipitant⁶². The crude preparation was desalted by gel filtration with Sephadex G-25 and applied to DEAE-cellulose column. The enzyme was eluted with a linear gradient of NaCl⁶². When the crude extract of enzyme was applied to DEAE-cellulose column, the dextranase activity could not be eluted from the column, even at very high ionic strengths⁶. This was because dextranase tends to aggregate in the presence of dextran. Most of the dextranase activity was found at the top one-third of the column. This could be eluted only at very high ionic strengths, and required extensive dialysis to remove the salt⁶. Monsan and Lopez reported a much higher specific activity and achieved the purification of dextranase from *L. mesenteroides* culture supernatant by slow addition of sucrose⁹. This resulted in high activity of enzyme that was purified by single step using gel permeation chromatography on Ultrogel AcA 34⁹. This procedure yielded enzyme with a specific activity of 122 U_{mg}⁻¹ with 96.4% yield. Kobayashi *et al.* obtained enzyme precipitates from *L. mesenteroides* culture supernatant by ethanol precipitation and centrifugation⁶³. The precipitate was put on DEAE-cellulose column and the enzyme was eluted with a NaCl gradient and the active fractions were pooled and concentrated. The enzyme was then put on Sephadex G-100 column and the active fractions collected, and a specific activity of 26 U_{mg}⁻¹ was obtained with 679-fold purification⁶³.

Dextranase treatment and chromatographic separation

Dextranases (EC 3.2.1.11) are enzymes, which cleave the glycosidic bonds in dextran. Dextranase is both glycosidic bond and strain specific. Dextranase is mostly obtained from fungi. Cleavage of dextran makes the enzyme purification easier as the culture broth becomes less viscous, facilitating the separation of multiple forms of enzyme. The culture supernatant of *L. mesenteroides* B-512 concentrate was loaded on Bio-Gel A-5m column, before and after dextranase treatment⁶. Dextranase and levansucrase migrated in the void volume before dextranase treatment. After treatment however, the dextranase was retarded by Bio-Gel A-5m, and levansucrase still migrated in the void volume. Furthermore, the added dextranase was retarded to a greater extent than dextranase, thus giving a separation of dextranase from levansucrase and dextranase. The purified dextranase was obtained with an activity of 53 U_{mg}⁻¹, yield of 33% and 99% of the protein recovered with 84% of the carbohydrate removed⁶. Chromatography on DEAE-cellulose after dextranase treatment removed

most of the polysaccharide associated with the enzyme⁶. The traces of carbohydrate left were removed after chromatography on Sephadex G-200. Sephadex G-200 had the highest affinity for dextranase (90 Uml⁻¹ hydrated gel) in comparison to other Sephadex matrices⁶. Dextranase treatment was required to degrade the enzyme associated dextran prior to DEAE-cellulose chromatography. Without dextranase treatment, up to 40% of the activity failed to bind to DEAE cellulose⁶. Traces of dextranase present in the dextranase preparation from DEAE-cellulose deteriorated the flow rates during chromatography on Sephadex due to degradation of Sephadex by dextranase. It therefore becomes essential to remove dextranase completely before applying the active fractions from DEAE-cellulose to Sephadex column.

Nitrosoguanidine mutant of *L. mesenteroides* B-512F with an activity of 3 Uml⁻¹ in the culture supernatant was selected for high dextranase production²⁴. The crude dextranase was incubated with immobilized dextranase. This preparation was passed over a column of Bio-Gel A-5m. The resulting purified enzyme lost activity rapidly but the enzyme preparations could be stabilized by low levels of high-molecular-weight dextran, polyethyleneglycol and nonionic detergents²⁴. An improvised protocol involving dextranase treatment, DEAE-cellulose chromatography, affinity chromatography on Sephadex G-200 and chromatography on DEAE-Trisacryl M to obtain dextranase with low carbohydrate content (1–100 µgmg⁻¹ protein) and relatively higher specific activity (90–170 Umg⁻¹) was developed¹¹. Levansucrase was completely removed during DEAE-cellulose chromatography and dextranase became undetectable after Sephadex G-200 chromatography¹¹. This method was also scaled up to produce gram quantities of purified enzyme. Kobayashi and Matsuda used sepharose 6B column for effective purification of dextranase after dextranase treatment⁶⁴. The culture supernatant of *L. mesenteroides* was added with slurry of hydroxyapatite which adsorbed the enzyme. The slurry was washed to desorb the enzyme that was applied to a column of Sephadex G-100. The major volume of enzyme, which had no affinity for sephadex, was eluted in the void volume fraction and digested with dextranase. After subsequent removal of the dextranase activity by CM-cellulose column, the enzyme fraction was chromatographed on a Sepharose 6B column. The sepharose 6B column was effective for the preparation of dextranase with no contaminating dextran and also removed traces of urea, which was used as elutant and resulted 72.1 Umg⁻¹ of specific activity⁶⁴. Crude enzyme preparation of *L. mesenteroides* B-512FM was treated with crude dextranase followed by column chromatography on Bio-Gel A-5m. The purified enzyme showed a specific activity of 84 Umg⁻¹¹².

Ultrafiltration

Bulk purification of extracellular dextranase from *L. mesenteroides* B-512F using ultra-filtration was attempted for commercial production of dextran⁶⁵. Ultracentrifugation and cross-flow ultrafiltration were employed in a three-stage process to obtain dextranase of over 95% purity with an overall activity of 60%⁶⁵. In the first stage, the cells were removed by continuous centrifugation at high centrifugal forces. This was followed by continuous ultra-filtration using hollow fiber membrane with flat cross flow to remove soluble low M_r impurities. The enzyme so obtained was stored with dextran as it prevented the loss of enzyme activity. Just before the use of enzyme for dextran synthesis, a second centrifugation process was carried out as the third stage process, to remove dextran⁶⁵.

A constitutive mutant *L. mesenteroides* B-512FMC-16, which produced 16 IUml⁻¹ dextranase with glucose in the medium, was used to obtain dextranase by ultra-filtration⁶⁶. The absence of dextran simplified the process facilitating large-scale production of highly pure dextranase by ultra-filtration. The process involved the addition of Tween 80 and CaCl₂ to the culture filtrate prior to concentration. Tween 80 dissociated inactive enzyme aggregates and produced active enzyme molecules. CaCl₂ caused aggregation of the enzyme molecules, which did not pass through a polysulfone ultrafiltration hollow fiber cartridge with 100 kDa cut-off at a constant flow rate resulting in concentration and purification. This was repeated to remove contaminating small molecules from the enzyme solution. The enzyme was concentrated from 23.50 L to 1.65 L, giving 280 IUml⁻¹ with only 7% loss in enzyme activity⁶⁶. Purified dextranase from *L. mesenteroides* B-512F was obtained by passing the culture supernatant through an ultra-filtration system, equipped with a 120 kDa cut off hollow fiber cartridge⁶⁷. Samples were lyophilized and re-suspended in buffer, containing CaCl₂ and sodium azide⁶⁷.

Purification of dextranase from constitutive mutants of *Leuconostoc mesenteroides*

The enzyme dextranase produced by *L. mesenteroides* mutants in the presence of various sugars was studied for adsorption of enzyme to the sephadex column⁶². Partial purification of enzyme was done by ammonium sulfate precipitation and passing through a column of hydroxyapatite⁶². The partially purified enzyme was run through sephadex column. The enzyme produced in glucose medium more readily adsorbed on sephadex G-100 and G-200, than that

produced in sucrose medium as the enzyme produced on glucose medium was free of any preformed dextran thus, eliminating the dextranase treatment and DEAE-cellulose separation. The adsorption by sephadex decreased when the glucose-produced enzyme was pre-incubated with dextrans of molecular size greater than 10 kDa, as the dextran gets associated with the enzyme and reduces the affinity of sephadex for enzyme⁶². The culture supernatant of constitutive mutant SH 3002 of *L. mesenteroides* B-512F grown on fructose was, put on DEAE-Sephadex A-50 column and eluted with NaCl gradient⁶⁸. The active fractions were freeze-dried and put on a Sepharose CL-6B column. A specific activity of 171 Umg⁻¹ with 43.8% activity yield was obtained⁶⁸. Purification of the enzyme from constitutive mutants has the advantage that it has no dextran along with the enzyme. This eases the chromatographic purification procedures by eliminating dextranase treatment and DEAE-cellulose chromatography.

Purification of recombinant dextranase

Commercially available dextranase produced from *L. mesenteroides* is induced by the addition of sucrose to the culture medium and this leads to enzyme preparations consisting of 99% dextran and only 1% protein. These preparations are difficult to use for the investigation of enzyme structure and catalysis. To obtain dextran free dextranase, recombinant dextranase of *Leuconostoc mesenteroides* B-742 production by expression in *E. coli* DH5 α cells was established and purified⁶⁹. The recombinant *E. coli* DH5 α cells were cultivated in Luria-Bertani medium containing 2% sucrose or glucose. The culture supernatant was concentrated by the addition of PEG 1500 to a final concentration of 20%. The extracellular activity was recovered in the dextran-rich phase. Dextran bound to the enzyme was removed by dextranase. The dextranase-treated enzyme was dialyzed and the dialyzate was loaded onto a DEAE-sepharose column. The enzyme from the column was eluted on a linear gradient of NaCl. The fractions with dextranase activity were pooled and applied to phenyl-sepharose column. The dextranase fraction was eluted with a linear gradient of decreasing ionic strength of sodium acetate buffer. A specific activity of 29.2 Umg⁻¹ was obtained⁶⁹.

The recombinant dextranase from *L. mesenteroides* B-512F was expressed and produced in *Bacillus megaterium* that has protein secretion capacity for the production for the production of exoenzymes²⁰. It was formed in the cytoplasm and released into the growth medium via its native leader sequence. For concentrating the excreted

recombinant dextranase from the growth medium, samples were frozen at -20°C to precipitate the protein and the enzyme was harvested by centrifugation. The induction in *Bacillus megaterium*, produced very high specific (362 Ug⁻¹) and volumetric (28,600 U l⁻¹) activity of dextran free dextranase²⁰. A recent report describes the production of recombinant *L. mesenteroides* B-512F dextranase with 6 His tag at the C-terminal and its expression in *E. coli* TOP10 cells¹⁶. The recombinant enzyme was purified by affinity chromatography using Ni²⁺ ion immobilized column. A highly pure dextranase was obtained with a specific activity of 430 Umg⁻¹¹⁶.

Conclusion

Dextranase is a commercially important enzyme. The most widely used organism, as a source of this enzyme is the *Leuconostoc* spp. because of the multiple applications of soluble dextran they produce. Dextranases in *Leuconostoc* are induced by sucrose, causing concomitant synthesis of dextran during enzyme production. The dextran so formed, leads to the formation of aggregated forms of dextranase and makes the growth medium viscous, making the enzyme purification difficult. Various methods like ultra-filtration, salt and PEG precipitation, chromatography, phase partitioning and a combination of these methods have been used to obtain pure enzyme. Dextranase dissociates the aggregated forms of dextranase. Dextranase treatment removes dextran from the enzyme, which simplifies the chromatographic processes, while dextran is required for efficient phase partitioning by PEG. Ultrafiltration systems with membranes of various pore sizes have been used for the large-scale purification of the enzyme with success. Polyethylene glycol of different molecular weights, have been used for the purification of enzyme by fractionation. It is a low cost, easy and rapid purification method. The dextranase treatment, followed by the removal of dextranase from dextranase, is inconvenient, expensive and time-consuming step, especially for the large-scale purification of dextranase. Moreover, some dextranases are rendered resistant to dextranase hydrolysis by contaminating non-hydrolysing dextran. Most of these difficulties are overcome by the use of constitutive mutants that can produce dextranase in the absence of sucrose. The procedures of purifying dextranase from *Leuconostoc* species either have low yields or fail to remove impurities, especially the associated polysaccharide. The advent of rDNA technology has led to the expression of enzyme in alternative hosts like *E. coli* and *Bacillus megaterium*. The enzyme is synthesized without

sucrose induction and released into the medium in large quantities. Consequently, the absence of contaminating dextran makes the enzyme purification method simpler.

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