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(54) **Title**: PROCESS FOR PURIFICATION OF MBP OR MBP TAGGED PROTEINS

(57) **Abstract**: A process for purifying a MBP or MBP tagged protein using alginate matrix is provided herein. The process and the composition are provided for increasing the binding affinity of a target protein and recovering a higher amount of the target protein. The present invention further provides alginate matrix for purification of the MBP or MBP tagged protein. The process for purifying a MBP or MBP tagged protein using alginate matrix as disclosed in the present invention results in recovery of more than 99% of the target protein.

PROCESS FOR PURIFICATION OF MBP OR MBP TAGGED PROTEINS

FIELD OF INVENTION

This invention relates generally to protein purification. In particular, the invention relates to a process for purifying a MBP or MBP tagged protein using alginate matrix and kits for use in the field of protein purification.

BACKGROUND OF THE INVENTION

Production of proteins/enzymes is an important area in biotechnology. Earlier, sources for obtaining proteins were limited to animals, plants, and microorganisms. In recent decades, cloning and tissue culture techniques have been increasingly used to source proteins. Purification of proteins involves separation of the desired protein from the complex mixture of other contaminating proteins and various low molecular weight compounds present in the initial crude preparation. Purification cost can constitute sometime even upto 90% of the total production cost. Traditionally, a protein purification protocol involves a primary stage wherein unit operations like precipitation, membrane based separation separates proteins from other kinds of material, concentrates this feed from the fermenter/crude extract, and also leads to a limited purification of the desired protein. For many industrial applications, this may be an adequate level of purification. For many applications, further secondary purification stage is required. This invariably involves gel filtration, ion exchange chromatography, and affinity chromatography as the final polishing stage. For pharmaceutical proteins such as vaccines and drugs, the protein preparation of highest purity level is desirable. Over the last few decades, few clear trends have emerged in the method development area for protein purification. Reducing the number of steps (i.e. unit processes) in the purification protocol is preferred as the net protein recovery decreases as the number of steps increase. Mass transfer limitations can be avoided by carrying out the separation in the free solution rather than in the chromatographic form. This is especially a valuable strategy in the context of affinity based separations [Przybycien T.M., Pujar N.S., Steele L.M. Alternative bioseparation operations: life beyond packed-bed

chromatography. Curr. Opin. Biotechnol. 15 (2004) 469]. Third trend is to develop methods which can directly deal with crude suspensions with suspended particles so that centrifugation (costly at large scale) is avoided. Aqueous two-phase system (ATPS), fluidized beds, three-phase partitioning (TPP), and macro-(affinity ligand) facilitated three-phase partitioning (MLFTPP) [Przybycien *et al.* (2005)] are some techniques which address this issue. Magnetic separation medium are especially useful in situations wherein fermenter feed/crude homogenate has high viscosity.

Affinity based separation methods generally offer much higher selectivity as compared to separation methods based upon other principles. In any affinity based separation method, a ligand having high affinity for the target protein is linked to an insoluble (affinity chromatography) or soluble matrix (affinity precipitation, aqueous two-phase affinity extractions and MLFTPP). In fortuitous cases, a matrix is available which itself has inherent affinity for the target proteins [Gupta M.N., Guoqiang D., Kaul R., Mattiasson B. Purification of xylanase from *Trichoderma viride* by precipitation with an anionic polymer Eudragit S 100. Biotechnol. Tech. 8 (1994) 117]. This saves the cost of the ligand and conjugation. This also leads to a safer protocol and more acceptable protocol by the regulatory agencies since the risk of ligand dissociating from the matrix during the process is eliminated. This is important if the end application of the protein is in food/pharma sectors.

Recombinant methods offer the possibility of producing the desired proteins with an 'affinity tag' (also called fusion tag) to produce the fusion protein. This enables the purification of the protein directly (skipping many steps) using an affinity based separation. The examples of popular fusion tags are polyhistidine, cellulose binding domain (CBD), Intein tag and MBP [Sachdev D., Chirgwin J.M. Fusions to maltose-binding protein: control of folding and solubility in protein purification, Methods Enzymol. 326 (2000) 312]. The core of this technology is designing or selecting a suitable affinity media which would capture the fusion protein via its interaction with 'fusion tag'. MBP as a fusion tag is recommended as it is claimed that a recombinant

protein produced with this tag is less likely to form "inclusion bodies". The latter are inactive solid particles often formed during overexpression of a recombinant protein in bacterial systems. This is believed to be due to aggregation of misfolded proteins. In such cases, the inclusion bodies have to be solubilized (by unfolding) and refolded to recover active soluble protein [Middelberg A.P.J. Preparative protein refolding. Trends Biotechnol. 20 (2002) 437]. In the case of MBP-tagged proteins, amylose [Riggs P.D., Hsieh P., Walker I., Colussi P.A., Ganatra M., Taron C.H. Solubilization and purification of a target protein fused to a mutant maltose-binding-protein. WO2007120809 (2007)], dextrin-sepharose [Cabanne C., Pezzini J., Joucla G., Hocquellet A., Barbot C., Garbay B., Santarelli X.. Efficient purification of recombinant proteins fused to maltose-binding protein by mixed-mode chromatography. J. Chromatogr. A 1216 (2009) 4451], cationic starch [Raghava S., Aquil S., Bhattacharyya S., Varadarajan R., Gupta M.N. Strategy for purifying maltose binding protein fusion proteins by affinity precipitation. J. Chromatogr. A 1194 (2008) 90], have been described as affinity media.

Using fusion tags like polyhistidine or maltose binding protein (MBP) is now a standard and widely used approach to purify recombinant proteins. The maltose-binding protein (MBP) is a 40.70 kDa, 370 amino acid, periplasmic protein of *E.* coli K12, involved in binding and transport of maltose and is encoded by the *malE* gene. The MBP can be fused at the N- or C-terminus of the protein if overexpression in bacteria is desired [Sachdev D., Chirgwin J.M. Fusions to maltose-binding protein: control of folding and solubility in protein purification. Methods Enzymol. 326 (2000) 312]. For purifying MBP-tagged proteins, two affinity matrices are commercially available viz. amylose resin by New England Biolabs and Dextrin sepharose by GE Healthcare.

Unlike above quoted commercially available matrices, alginate can be viewed as a smart (Ca^{2+} responsive), a stimuli sensitive or reversible soluble insoluble polymer. Hence a non chromatographic approach called affinity precipitation [Mondal K., Gupta M.N., Roy I. Affinity-based strategies for protein purification. Anal. Chem. 78 (2006a)

3499] can be used for purifying proteins. Affinity precipitation is a scalable inexpensive batch process with many advantages [Mondal *et al.* (2006a)] over chromatographic methods.

US patent 5643758 describes a method for producing and/or purifying any hybrid polypeptide molecule employing recombinant DNA techniques. The method as described involves expression of hybrid polypeptide comprising a binding protein that can be purified by contacting the hybrid polypeptide with a ligand or substrate to which the binding protein has specific affinity for example affinity chromatography.

Alginate is known to bind some carbohydrate binding enzymes [Teotia S., Khare S.K., Gupta M.N. An efficient purification process for sweet potato beta-amylase by affinity precipitation with alginate. Enzyme Microb. Tech. 28 (2001) 792] and can be used in affinity based separations in packed bed [Jain S, Gupta MN. Purification of goat immunoglobulin G by immobilized metal-ion affinity using cross-linked alginate beads. Biotechnol. Appl. Biochem. 39 (2004) 319], fluidized bed [Roy I., Jain S., Teotia S., Gupta M.N. Evaluation of Microbeads of Calcium Alginate as a Fluidized Bed Medium for Affinity Chromatography of *Aspergillus niger* Pectinase. Biotechnol. Prog. 20 (2004) 1490], two phase mode [Teotia S., Gupta M.N. Reversibly soluble macroaffinity ligand in aqueous two-phase separation of enzymes. J Chromatogr A 923 (2001) 275] and magnetic mode [Šafařiková M., Roy I., Gupta M.N., Šafařik I. Magnetic alginate microparticles for purification of α-amylases. J. Biotechnol. 105 (2003) 255] as well.

Thus, there is a need to produce recombinant proteins in a highly purified and well characterized form and exhibiting native conformation. In other words there is need to provide a solution for problems associated with the purification and yield of target protein. The present invention provides a solution to this problem by providing a process of purification of proteins whereby all contaminating proteins or impurities are at least partially, preferably completely, removed in a single purification step. The invention also provides for a simultaneous refolding strategy in case the starting preparation is in inactive form including inclusion bodies.

SUMMARY OF THE INVENTION

It is one of the aspect of the present invention to achieve high yield of a target protein or protein of interest which is substantially pure with no or negligible amount of impurities.

Another aspect of the present invention provides a protein purification process comprising contacting a sample comprising a target protein with a matrix comprising 0.1% to 2.0% (w/v) alginate to obtain a protein-alginate complex, wherein the target protein is MBP or MBP tagged protein; separating the protein-alginate complex, and purifying the target protein from the protein-alginate complex.

Yet another aspect of the present invention provides a protein purification process, comprising permitting a sample comprising a target protein to bind to a matrix comprising 0.1% to 2.0% (w/v) alginate, wherein the target protein is MBP or MBP tagged protein; and purifying the target protein from the matrix in a selected buffer to obtain the purified target protein.

Still another aspect of the present invention provides a process for purifying a target protein, wherein the process comprises providing a DNA expression vector capable of expressing a target protein in a host cell, wherein the target protein is tagged with MBP; transforming a host cell with the DNA expression vector and expressing the target protein; contacting the target protein with a matrix comprising 0.1% to 2.0% (w/v) alginate to obtain a protein-alginate complex, separating the protein-alginate complex, and purifying the target protein from the protein-alginate complex, wherein purified target protein is capable of refolding into their native conformation.

Still yet another aspect of the present invention provides a device for selective binding and separation of a MBP or MBP tagged target protein from a sample, wherein the device comprising housing, an inlet, an outlet and at least one separation matrix comprising alginate or alginate beads.

Further aspect of the present invention is to provide a protein purification kit comprising the device comprising housing, an inlet, an outlet, and at least one separation matrix comprising alginate or alginate beads, a precipitation and washing buffer comprising $CaCl_2$, and an elution buffer comprising maltose or a suitable eluent.

BRIEF DESCRIPTION OF ACCOMPANYING DRAWINGS

Figure 1 shows SDS-PAGE analysis of the MBP and MBP fusion proteins purified by alginate.

(A) Fifteen percent SDS-PAGE analysis showing purification of WT-MBP by alginate; (lane 1) molecular weight markers; (lane 2) crude WT-MBP obtained after osmotic shock treatment of cells; (lane 3) purified WT-MBP.

(B) Twelve percent SDS-PAGE analysis showing purification of MBP-H3HA9 fusion protein by alginate; (lane 1) molecular weight markers; (lane 2) crude cell lysate containing MBP-H3HA9 fusion protein; (lane 3) purified MBP-H3HA9 fusion protein.

(C) Twelve percent SDS-PAGE analysis showing purification of MBP-CD4bs fusion protein by alginate; (lane 1) molecular weight markers; (lane 2) crude cell lysate containing MBP-CD4bs fusion protein; (lane 3) purified MBP- CD4bs fusion protein.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The terms "protein," "polypeptide" and "peptide" as used herein refer to a polymer of amino acid residues. The terms also apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers, those

containing modified residues, and non-naturally occurring amino acid polymer. Peptides are distinguished by the number of amino acid residues making up the primary structure of the molecule. For purpose of this invention, typically, peptides are those molecules comprising up to 50 amino acid residues and proteins comprise more than 50 amino acid residues.

The terms "fusion protein", and "target protein" used herein can be used interchangeably.

The terms "MBP tagged Protein" and "MBP tagged fusion Protein" used herein can be used interchangeably.

"Purifying" a target protein from a sample comprising the target protein and one or more contaminating proteins or impurities refers to increasing the degree of purity of the target protein by removing partially or completely at least one or more of the contaminating proteins or impurities.

The term "Recombinant protein" or "fusion protein" as used herein refers to a protein which has been produced in a host cell which has been transformed or transfected with nucleic acid encoding the protein, or produces the protein as a result of homologous recombination.

The term "Sample" as used herein refers to any composition, preferably an aqueous solution that comprises a target protein group of interest and contaminating proteins and is in a physical state which allows any target protein group of interest and any contaminating protein present in the sample to be contacted with a library of binding moieties. Samples may be of any source that comprises MBP or MBP tagged target protein of interest.

The term "Solid support" as used herein refers to any insoluble material including particles (e.g., beads), fibers, monoliths, membranes, filters, plastic strips, and the like.

A protein can exist in a sample in several forms that can be co-purified. The term "target protein" as used herein refers to a single protein or group of related proteins to

be purified. These related forms can result from either, or both, of pre-and post-translational modification. Pre-translational modified forms include allelic variants, slice variants, and RNA editing forms. Post-translationally modified forms include forms resulting from proteolytic cleavage (e.g., fragments of a parent protein), glycosylation, phosphorylation, lipidation, oxidation, methylation, cystinylation, sulphonation and acetylation.

The term "composites of alginate" reefers to an alginate matrix comprising one or more composite material including chitosan, starch, cellulose, dextran, carrageenan, proteins, gelatin, oxides, SiO_2, heparin, Hyaluronic acid or combinations thereof.

The collection of proteins including a specific protein and all modified forms of it is referred to herein as "a protein of interest." Thus, for example, albumin and modified forms of albumin found in serum are a target protein. Furthermore, a protein may be expressed as a multimeric protein, such as a dimeric protein. Examples of this are immunoglobulins and insulin. The term "a protein of interest" embraces this as well.

The present invention provides a novel matrix for MBP or MBP tagged target protein, wherein the novel matrix is comprised of alginate. More particularly, the present invention provides a process for purifying virtually any MBP tagged recombinant protein using the alginate matrix that results in recovery of about 96% to 99.9% of the target protein.

The protein purification process as disclosed in the present invention does not employ costly reagents with their attendant disposal problems, does not require multiple steps, and results in high yield of a pure, biologically active protein product.

The present invention provides a process for obtaining highly purified virtually any protein molecule produced by recombinant technology in a single step or if necessary another chromatographic step followed by it, wherein the protein is tagged with MBP. In particular the present invention provides a process for purifying a target protein in the form of fusion protein comprising MBP as a binding protein produced by recombinant DNA technology. The fusion protein can be isolated and purify directly from the crude

extract or culture medium by contacting the extract or the culture medium comprising the fusion protein to the alginate matrix as disclosed in the present invention.

The purified target protein comprising the binding protein can be further processed to separate the binding protein. It can be performed by various methods known in the art such as using a linker DAN sequence for linking a DNA encoding binding protein and a DNA encoding protein of interest to obtain a fusion protein.

The MBP or MBP tagged target protein comprising the a protein of interest is produced by constructing a cloning vector containing fused genes comprising a gene encoding a protein molecule of interest and a gene coding for a MBP or a portion or a variant thereof which has a specific affinity for alginate and expressing the fusion in suitable host cell such as *E. coli*, yeast, animal cell, insect cell or plant cell.

The protein purification processes as described herein by which DNA coding for a fusion protein comprising a protein of interest and MBP or a portion or a variant thereof is preferably cloned, expressed and purified using the alginate matrix as disclosed in the present invention. The process comprises constructing a recombinant fusion DNA expression cassette, wherein the DNA expression cassette comprises the polynucleotide encoding MBP or fragment thereof or a variant thereof and a polynucleotide encoding the protein of interest operably linked to a promoter, producing a recombinant vector comprising the recombinant fusion DNA expression cassette, transforming the recombinant vector into an appropriate host such as *E. coli*, selecting the transformants with antibiotic selection or other phenotypic selection, expressing the fusion protein in an appropriate host cell such as *E. coli*, yeast, animal cell, insect cell or plant cell and purifying the fusion protein comprising the MBP or MBP tagged by contacting crude protein sample or the culture medium potentially comprising the target protein with a matrix comprising 0.1% to 2.0% (w/v) preferably 0.5% (w/v) alginate to obtain a protein-alginate complex, separating the protein-alginate complex by adding $CaCl_2$ solution to a final concentration of 0.06M, and purifying the target protein from the protein-alginate complex by adding $CaCl_2$ solution to a final concentration of 0.06M.

In accordance with the present invention, in one embodiment there is provided a protein purification process comprising contacting a sample comprising a target protein with a matrix comprising 0.1% to 2.0% (w/v) alginate to obtain a protein-alginate complex, wherein the target protein is MBP or MBP tagged protein; separating the protein-alginate complex, and purifying the target protein from the protein-alginate complex.

The protein purification process as disclosed in the present invention is applicable to industrial-scale purification as a new purification procedure.

In another embodiment of the present invention there is provided a protein purification process comprising contacting a sample comprising a target protein with a matrix comprising 0.5% (w/v) alginate to obtain a protein-alginate complex, wherein the target protein is MBP or MBP tagged protein; separating the protein-alginate complex, and purifying the target protein from the protein-alginate complex.

In another embodiment of the present invention there is provided a protein purification process comprising contacting a sample comprising a target protein with a matrix comprising 0.5% (w/v) alginate to obtain a protein-alginate complex, wherein the target protein is MBP or MBP tagged protein; separating the protein-alginate complex, and purifying the target protein from the protein-alginate complex, wherein the matrix further comprises one or more composite material selected from a group consisting of chitosan, starch, cellulose, dextran, carrageenan, proteins such as gelatin, oxides, SiO_2, heparin, and Hyaluronic acid.

In another embodiment of the present invention there is provided a protein purification process comprising contacting a sample comprising a target protein with a matrix comprising 0.1% to 2.0% (w/v) alginate to obtain a protein-alginate complex, wherein the target protein is MBP or MBP tagged protein; separating the protein-alginate complex by adding $CaCl_2$ solution to a final concentration of 0.06M, and purifying the target protein from the protein-alginate complex.

In another embodiment of the present invention there is provided a protein purification process comprising contacting a sample comprising a target protein with a matrix

comprising 0.1% to 2.0% (w/v) alginate to obtain a protein-alginate complex, wherein the target protein is MBP or MBP tagged protein; separating the protein-alginate complex, washing of the protein-alginate complex, and purifying the target protein from the protein-alginate complex.

In yet another embodiment of the present invention there is provided a protein purification process comprising contacting a sample comprising a target protein with a matrix comprising 0.1% to 2.0% (w/v) alginate to obtain a protein-alginate complex, wherein the target protein is MBP or MBP tagged protein; separating the protein-alginate complex, washing of the protein-alginate complex of step (b) by adding $CaCl_2$ solution to a final concentration of 0.06M, and purifying the target protein from the protein-alginate complex.

In yet another embodiment of the present invention there is provided a protein purification process comprising contacting a sample comprising a target protein with a matrix comprising 0.1% to 2.0% (w/v) alginate to obtain a protein-alginate complex, wherein the target protein is MBP or MBP tagged protein; separating the protein-alginate complex, and purifying the target protein from the protein-alginate complex by adding 1M Maltose solution at 4°C to 25°C preferably at 4°C for 1-24 hours preferably for 18 hours.

In one of the preferred embodiment of the present invention there is provided a protein purification process comprising contacting a sample comprising a target protein with a matrix comprising 0.5% (w/v) alginate at 25°C±2 to obtain a protein-alginate complex, wherein the target protein is MBP or MBP tagged protein; separating the protein-alginate complex by adding $CaCl_2$ solution to a final concentration of 0.06M, washing the protein-alginate complex with a buffer comprising 0.06M $CaCl_2$ and purifying the target protein from the protein-alginate complex by adding 1M Maltose solution at 4°C to 25°C preferably at 4°C for 1-24 hours preferably for 18 hours.

Yet another embodiment of the present invention provides a protein purification process comprising permitting a sample comprising a target protein to bind to a matrix

comprising 0.1% to 2.0% (w/v) alginate, wherein the target protein is MBP or MBP tagged protein; and purifying the target protein from the matrix in a selected buffer to obtain the purified target protein.

Still another embodiment of the present invention provides a process for producing and purifying a target protein, wherein the process comprises producing the target protein by transforming a host cell with a recombinant vector comprising a DNA expression cassette comprising the polynucleotide encoding the target protein, wherein the target protein is tagged with MBP; contacting the target protein with a matrix comprising 0.1% to 2.0% (w/v) alginate to obtain a protein-alginate complex, separating the protein-alginate complex, and purifying the target protein from the protein-alginate complex.

Still another embodiment of the present invention provides a process for producing and purifying a recombinant MBP protein, wherein the process comprises producing the recombinant MBP protein in a host cell by transforming the host cell with a recombinant vector comprising a DNA expression cassette comprising a polynucleotide encoding the recombinant MBP (maltose-binding protein) protein, contacting the recombinant MBP protein with a matrix comprising 0.5% (w/v) alginate to obtain a MBP protein-alginate complex, separating the MBP protein-alginate complex, and purifying the recombinant MBP protein from the MBP protein-alginate complex.

Still another embodiment of the present invention provides a process for producing and purifying a recombinant MBP tagged CD4bs (a fragment of HIV protein gp120) fusion protein, wherein the process comprises producing the recombinant MBP tagged CD4bs fusion protein in a host cell by transforming the host cell with a recombinant vector comprising a DNA expression cassette comprising a polynucleotide encoding the recombinant MBP tagged CD4bs fusion protein, contacting the recombinant MBP tagged CD4bs fusion protein with a matrix comprising 0.5% (w/v) alginate to obtain a MBP tagged CD4bs-alginate complex, separating the MBP protein-alginate complex,

and purifying the MBP protein tagged CD4bs fusion from the MBP tagged CD4bs-alginate complex.

Still another embodiment of the present invention provides a process for producing and purifying a recombinant MBP tagged H3HA9 (one of the influenza hemagglutinin) fusion protein, wherein the process comprises producing the recombinant MBP tagged H3HA9 fusion protein by transforming a host cell with a recombinant vector comprising a DNA expression cassette comprising a polynucleotide encoding the MBP tagged H3HA9 fusion protein, contacting the MBP tagged H3HA9 fusion protein with a matrix comprising 0.5% (w/v) alginate to obtain a MBP tagged H3HA9-alginate complex, separating the MBP protein-alginate complex, and purifying the MBP tagged H3HA9 fusion protein from the MBP tagged H3HA9-alginate complex.

One embodiment of the present invention relates to a host cell for production of recombinant MBP or MBP tagged fusion protein, wherein the host cell is a bacterial cell such as *E. coli* or yeast or fungal cell or mammalian cell or plant cell or a similar organism capable of producing the protein.

Surprisingly, it was observed that the alginate matrix based protein purification method as disclosed in the present invention results in higher yield of pure MBP or MBP tagged target protein. The alginate matrix based protein purification method of the present invention purifies at least 96% of the target protein is purified, more preferably at least 97%, most preferably at least 98%. Most surprisingly it was found that the alginate matrix based protein purification method for purification of MBP tagged target protein of the present invention can purify up to 99% to 99.9% of the target protein.

The process of protein purification as disclosed in the present invention is able to purify more than 98% preferably mere that 99% of target protein which is in the form of a soluble protein or in the form of inclusion body. In case of inclusion bodies, surprisingly it was found out that the protein in the form solubilized inclusion bodies purified using the alginate matrix as disclosed in the present invention is capable of refolding into their native conformation.

If the MBP tagged fusion protein is expressed as inactive and insoluble inclusion bodies, then refolding and simultaneous purification of the protein can be carried out by alginate matrix as disclosed in the present invention from the inclusion bodies. In case refolding along with purification is desired from the inclusion bodies then before refolding and purification, solubilization of inclusion bodies in 8 M urea or 6 M GdmCl (guanidium chloride) or by any other means is carried out.

The process for protein purification as disclosed in the present invention can be carried out in following modes:

Batch mode: By precipitation of the protein-alginate complex by adding 0.06 M CaCl2 and then elution/dissociation of protein-polymer complex by maltose or by any other suitable means (depending upon the target protein). Here alginate concentration can be generally varied from 0.1% to 5%.

Packed bed column mode: A chromatographic column is packed with alginate beads and the purification is carried out as affinity chromatography. Here the alginate beads are packed in a column and then the purification is carried out.

Expanded (stable fluidized) bed column mode: Purification is carried out in expanded (stable fluidized) bed column chromatography mode by using alginate beads. Here the alginate beads are loaded on a fluidized bed column.

Magnetic mode: Alginate can be converted into magnetic beads by coating of particles (such as Fe_3O_4 particles) of any size (for nanoparticles, micron size particles etc.) with alginate and then magnetic separation of protein-alginate (alginate is magnetic) complex by the means of a magnet.

Elution or dissociation of the purified MBP tagged fusion protein from the alginate in all the above modes was carried out by using 1 M Maltose, wherein the concentration of Maltose is in the range of 0.05 M to 2 M.

The final purity of the purified protein obtained using the alginate matrix as disclosed in the present invention is more than 98% or up to 99.9% as observed by densitometer

analysis of SDS-PAGE, which is even higher as compared to any known process of protein purification using Dextrin Sepharose matrix (GE Healthcare) which yields 95% purity by SDS-PAGE.

Another embodiment of the present invention provides a device for selective binding and separation of a MBP or MBP tagged target protein from a sample, wherein the device comprising housing, an inlet, an outlet and at least one separation matrix comprising alginate or alginate beads.

Another embodiment of the present invention related the device as disclosed in the present invention, wherein the device comprises alginate matrix in the form of alginate beads.

Another embodiment of the present invention related the device as disclosed in the present invention, wherein the device comprises alginate matrix in the form of magnetic alginate beads.

Another embodiment of the present invention related the device as disclosed in the present invention, wherein the device is packed bed column or fluidized bed column.

The alginate can be converted into magnetic beads by coating of particles (such as Fe_3O_4 particles) of any size (for nanoparticles, micron size particles etc.) with alginate and then magnetic separation of protein-alginate (alginate is magnetic) complex by the means of a magnet.

The present invention further provides a matrix comprising alginate (0.1% to 2%) for purification of MBP or MBP tagged protein, wherein the matrix further comprises composite materials including polysaccharides e.g. chitosan, starch, cellulose, dextran and carrageenan; proteins, e.g. gelatin; oxides e.g. SiO_2 as a core or coat material, heparin, Hyaluronic acid etc.

Further, the alginate matrix beads as disclosed in the present invention particles can be of various sizes including nanodimensions made up of either alginate alone or in combination with composite materials including polysaccharides e.g. chitosan, starch,

cellulose, dextran and carrageenan; proteins, e.g. gelatin; oxides e.g. SiO_2 as a core or coat material, heparin, Hyaluronic acid etc.

Yet another embodiment of the present invention provides a protein purification kit comprising housing, an inlet, an outlet, and at least one separation matrix comprising alginate or alginate beads, a precipitation and washing buffer comprising $CaCl_2$, and an elution buffer comprising Maltose or a suitable eluent.

The inventors of the present invention unexpectedly found out that about 99 to 99% MBP tagged target protein can be obtained using alginate matrix in purification process and thus the simple alginate which is an inexpensive, non-toxic food grade polysaccharide of marine origin emerged as more useful matrix than existing options of amylase resin and dextrin sepharose.

Further, if MBP-tagged proteins are produced as inactive inclusion bodies, alginate based strategies (precipitation, packed bed, fluidized bed etc.) will be able to simultaneously refold the fusion proteins as well. This is obvious from the earlier results wherein alginate refolded urea denatured amylase [Mondal K., Raghava S., Barua B., Varadarajan R., Gupta M.N. Role of stimuli-sensitive polymers in protein refolding: α-Amylase and CcdB (controller of cell division or death B) as model proteins, Langmuir 23 (2007) 70].

One embodiment provides a process of purification of MBP or MBP tagged recombinant protein using the alginate matrix and suitable buffers, wherein the alginate matrix is in form of beads. The alginate beads matrix of the present invention can be in form of magnetic beads.

Another embodiment of the present invention provides a process of purification of MBP or MBP tagged target recombinant protein using alginate solutions (0.1% to 2%) and polymer such as PEG as one phase and polymer/salt solution of as another phase for carrying out separation of the target recombinant protein using Aqueous two-phase systems.

Another embodiment of the present invention provides a process of purification of MBP or MBP tagged target recombinant protein using alginate solutions (0.1% to 2%) and polymer such as PEG as one phase and polymer/salt solution of as another phase for carrying out separation of the target recombinant protein using Aqueous two-phase systems.

An aqueous two-phase system (ATPS) forms when two types of water soluble polymers or a water soluble polymer and a low molecular weight substance (inorganic salt in general) dissolve in aqueous solution above their critical concentrations. The top phase is rich in one polymer, and the bottom phase is rich in the other polymer or the salt. Both the two phases contain water at high proportion (about 80~99% by weight), and possess extremely low interfacial tensions. ATPSs provide different physical and chemical environments which allow for the partitioning of biomolecules such as proteins, plasmid DNA, toxin and so on. Various compositions including polymer/polymer, polymer/salt, surfactants, are important examples for ATPS in protein separation.

Another embodiment of the present invention provides a process of purification of MBP or MBP tagged target recombinant protein using alginate solutions (0.1% to 2%) using multi-phase systems.

Multiphasic systems consist of more than two phases. Important examples of such systems are TPP systems or MLFTPP systems. In the latter, alginate-target protein complex are known to separate as interfacial layer when appropriate amounts of a salt such as ammonium sulphate and an organic solvent (such as t-butanol) is mixed to a crude extract of protein [Sharma A., Roy I., Gupta M.N. Affinity precipitation and macroaffinity ligand facilitated three-phase partitioning for refolding and simultaneous purification of urea-denatured pectinase. Biotechnol. Progr. 20 (2004) 1255]. As is shown in the above reference, alginate can again simultaneously refold even in such systems.

EXAMPLES

It should be understood that the following examples described herein are for illustrative purposes only and that various modifications or changes in light will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

Materials

Protanal LF 10/60 (free alginate from brown seaweed) having a high content of guluronic acid (65-75%) was a product of Protan A/S (Drammen, Norway). The average molecular weight of Protanal LF 10/60 is 3,20,000 g/mol [Amsden B., Turner N. Diffusion characteristics of calcium alginate gels. Biotechnol. Bioeng. 65 (1999) 605]. PMSF (phenylmethanesulfonyl fluoride) and ampicillin were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). All the other chemicals were of analytical reagent quality.

Plasmids & Strains

E. coli DH5α was used for wild type (WT) MBP and BL21 (DE3) for MBP fusion proteins with CD4bs and H3HA9.

Example 1

Over-expression of WT-MBP /MBP tagged protein in *E. coli*

The plasmid pMALp2 expressing WT-MBP was transformed into *E. coli* DH5α. A single colony was picked and inoculated into 5 mL LB medium containing 100 $\mu g/mL^{-1}$ ampicillin. The tubes were shaken overnight at 37°C at 200 rpm. One percent of primary inoculum was transferred into 1 L fresh LB broth (amp+) and grown at 37°C with vigorous shaking until OD_{600} reached 0.8. Induction was carried out by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and the culture was further grown under similar conditions for 12 h at 37°C at 200 rpm.

This procedure was repeated for the transformation of the plasmid pET-22b-HMGWA containing MBP-CD4bs fusion protein into *E. coli* BL21 (DE3) and expressed.

The plasmid pET-22b(+) expressing MBP-H3HA9 fusion protein was transformed into *E. coli* BL21 (DE3) and the culture was grown culture at 37°C with vigorous shaking until OD_{600} reached 0.6-0.8 and the culture was further grown at 25°C for 12 h at 200 rpm after induction with 1 mM IPTG.

Example 2

Isolation of WT-MBP/ MBP tagged Proteins from *E. coli*

The cells were harvested by centrifugation at 8000×*g* for 10 min at 4°C. Crude MBP was isolated from *E. coli* cells employing the osmotic shock procedure [Ganesh C., Shah A.N., Swaminathan C.P., Surolia A., Varadarajan R. Thermodynamic Characterization of the reversible, two-state unfolding of maltose-binding protein, a large two-domain protein. Biochemistry 36 (1997) 5020]. The cells were washed twice with one-tenth of the culture volume of OSF I (10 mM Tris, 30 mM NaCl, and 100 μM PMSF at pH 7.2). The washed pellet was then resuspended in one twentieth of the culture volume of OSF II (30 mM Tris, 0.1 mM EDTA, 100 μM PMSF, and 20% w/v sucrose at pH 7.2), stirred at room temperature for 20 min, and then pelleted at 8500g for 20 min. The cell pellet was osmotically shocked by resuspending the cells in one-tenth of the culture volume of ice-cold OSF III (100 μM MgCl2 and 100 μM PMSF) and stirred at 4°C for 20 min. The suspension was centrifuged at 11000g for 20 min, and the supernatant so obtained is the OSF. Tris-HCl, MgCl2, and PMSF were added to final concentrations of 10 mM, 2 mM, and 100 μM, respectively, and the pH was adjusted to 7.5 at 4°C.

MBP-H3HA9 and MBP-CD4bs fusion proteins were isolated from *E. coli* cells by sonication in 50 mM Tris-HCl buffer, pH 7.5, containing 100 μM PMSF, 10 times with 30 s pulses on ice, and centrifugation at 9000×*g* for 30 min at 4°C. The supernatant thus obtained was used as crude extract for MBP-CD4bs and MBp-H3HA9 fusion proteins.

Example 3

Preparation of Alginate Beads (Beads, Microbeads and Cross-Linked Beads)

Alginate beads were prepared as described earlier [Roy *et al.* (2004)]. Beads were formed by dropping 50 mL of 2% (w/v) alginate solution through a syringe into 100 mL of 0.1 M $CaCl_2$ solution. The beads obtained were kept for 2 h in 0.1M $CaCl_2$ solution and stored in a 0.006M $CaCl_2$ solution at 4 °C.

The alginate microbeads were also prepared as described previously [Roy *et al.* (2004)]. 100 mL of alginate solution (2%, w/v) was taken in a handheld glass sprayer (normally used for spraying chromatograms) (16), one end of which was connected to the air cylinder. Alginate solution was sprayed into 500 mL of 1 M CaCl2 solution, at a constant pressure of 2 kg cm-2. The microbeads formed were filtered after 2 h on a Bu¨ chner funnel under vacuum and dried overnight in the oven at 50 °C.

Cross-linked alginate beads were prepared as described earlier [Sharma S., Roy I., Gupta M.N. Separation of phospholiase D from peanut on a fluidized bed of crosslinked alginate beads. Biochem. Eng. J. 8 (2001) 235]. Alginate was added to an alcoholic solution of epichlorohydrin (3 ml of epichlorohydrin in 15 ml of 95% ethanol). A total of 5 ml of 5N NaOH was added to this mixture and the suspension was gently rotated at 50 rpm for 8 h on a rotatory evaporator bath kept at 40 °C. Thereafter, the mixture was neutralized to pH 7.0 with 1M acetic acid. The cross-linked alginate beads thus formed were washed with 30 ml of a 3:1 mixture (v/v) of absolute ethanol and water, followed by 20 ml of 95% ethanol. The matrixwas left to dry to constantweight at room temperature. The dried material was resuspended in 50 ml of distilled water, stirred and left to dry at constant temperature. The suspension was decanted and the material remaining at the bottom of the beaker was collected on a coarse sintered glass filter, washed with 20 ml of 95% ethanol, dried again at room temperature to constant weight. The matrix was finally resuspended and equilibrated in a suitable buffer for 30 min before use.

Example 4

Preparation of Magnetic Alginate Beads

Alginate solution (20 ml, 2% w/v) was taken into 80 ml of Fe_3O_4 nanoparticles (0.5 g/100 ml) suspensions, the mixture was stirred at 50 °C for 45 min, and then the coated nanoparticles were separated by the means of a magnet and washed with double distilled water.

Example 5

Purification of WT-MBP/ MBP Fusion Proteins with Alginate

Preparation of Alginate Solution

Alginate solution (2%, w/v) was prepared in distilled water by dissolving 1 g of alginate in 50 mL of water. The solution was stored at 4°C and diluted with appropriate buffer for further use [Mondal K., Bohidar H.B., Roy R.P., Gupta M.N. Alginate-chaperoned facile refolding of *Chromobacterium viscosum* lipase. Biochim. Biophys. Acta. 439 (2006b) 1017].

Purification by Affinity Precipitation

Different aliquots of the crude extracts of overexpressed WT-MBP, MBP-CD4bs and MBP-H3HA9 fusion proteins (final protein concentration was 0.50 to 3.00 mg/mL^{-1}) were incubated with 0.5 mL of 2% (w/v) alginate (final concentration, 0.5%, w/v), and the final volume was made up to 2 mL with 0.05 M Tris-HCl buffer, pH 7.5. After incubation at 25°C for 1 h, the alginate-protein complex was precipitated by the addition of 1 M $CaCl_2$ (final concentration of $CaCl_2$ in solution was 0.06 M). The precipitate was separated from the supernatant by centrifugation (10000×*g*, 10 min). The precipitate was then washed twice with 2 mL of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.06 M $CaCl_2$. The bound protein was eluted off of the alginate by suspending the alginate-protein complex in 2 mL of chilled 1 M Maltose (prepared in 50 mM Tris-HCl buffer, pH 7.5) and incubating this suspension at 4°C for 18 h.

Purification using Aqueous Two-Phase Systems (ATPS)

Phase systems were prepared in graduated centrifuge tubes by mixing 22% (w/v) PEG 6000, 10% (w/v) dipotassium hydrogen orthophosphate, 10% (w/v) sodium chloride. The two distinct phases were formed within 5 min. To this aqueous two-phase system 0.5% (w/v) alginate was incorporated. Thereafter, crude preparations of WT-MBP, MBP-CD4bs and MBP-H3HA9 fusion proteins (1.0 ml) were added to the corresponding systems. Alginate distribution was restricted to the PEG phase with less than 5% (of the initially added amount) going to the bottom phase. Alginate concentration in the two phases was estimated by the phenol–sulphuric acid method [Hirs C.H.W. Glycopeptides. Methods Enzymol. 11 (1967) 411]. The two phases were separated. The alginate in the top phase was precipitated in the presence of 0.07M Ca^{2+} and incubated for 20 min at 25°C. The precipitate was centrifuged at 8000 *g* for 10 min at 25°C. The supernatant and subsequent washings with buffer (until no protein was detected in the washings) were collected. The amount of partitioned protein was calculated by the difference of initial activities and activities recovered in supernatant and washings.

Purification using Macro-(affinity ligand) Facilitated Three-Phase Partitioning (MLFTPP)

Various amounts of WT-MBP, MBP-CD4bs and MBP-H3HA9 fusion proteins were added to 1ml alginate (0.5%, w/v). The final volume was made up to 2 ml with 0.05M Tris-HCl buffer, pH 7.0. The protein containing solution was made up to 30% (w/v) with respect to ammonium sulfate and 4ml t-butanol was added. Gentle vortexing was followed by incubating the systems at 37°C for 1 h. Formation of three phases (upper organic phase, interfacial precipitate, and lower aqueous phase) was observed. The upper t-butanol layer was removed carefully with a Pasteur pipette. After this, the lower aqueous layer was removed by piercing the interfacial precipitate layer using another Pasteur pipette. The difference between the total protein in the crude extract and the protein in the aqueous phase represented the amount of protein bound to the alginate in

the interfacial layer. The interfacial precipitate consisting of alginate bound protein was dissolved in 3 ml of 1M maltose and incubated at 4°C for 4 h. Protein was then recovered by precipitating the alginate with 0.21 ml of 1M $CaCl_2$ (the final concentration of $CaCl_2$ in the solution was 0.07M). Protein activity and the protein concentration in the supernatant were determined after extensive dialysis to remove maltose.

Example 6

Protein Estimation

Protein concentration was estimated by the dye binding method using bovine serum albumin as the standard protein [Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1976) 248].

Polyacrylamide Gel Electrophoresis

SDS-PAGE of the protein samples using 15% gel was performed according to Laemmeli [Laemmeli U.K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 227 (1970) 680], using a Genei gel electrophoresis unit (Bangalore Genei, Bangalore, India). The protein bands on the gel were visualized using Coomassie Blue stain.

Table 1: Purification of WT-MBP & MBP-CD4bs fusion proteins by affinity precipitation with alginate

Protein	**Total protein**	**Total MBP/MBP tagged Fusion Protein***	**Bound Protein**	**Unbound Protein**	**Eluted Protein**	**Eluted MBP/ MBP tagged Fusion Protein**

WT-MBP	5.0 mg (100%)	3.4 mg (68%)	3.7 mg (74%)	1.3 mg (26%)	3.4 mg (92%)[a] (68%)[b]	> 99%
MBP-H3HA9	5.0 mg (100%)	3.0 mg (60%)	3.3 mg (66%)	1.7 mg (34%)	3.0 mg (91%)[a] (60%)[b]	> 99%
MBP-CD4bs	5.0 mg (100%)	2.4 mg (48%)	2.8 mg (56%)	2.2 mg (44%)	2.3 mg (82%)[a] (46%)[b]	96%

*by densitometer analysis

[a]with respect to bound protein

[b]with respect to total protein

We Claim:

1. A protein purification process comprising
    a. contacting a sample comprising a target protein with a matrix comprising 0.1% to 2.0% (w/v) alginate to obtain a protein-alginate complex, wherein the target protein is MBP or MBP tagged protein;
    b. separating the protein-alginate complex, and
    c. purifying the target protein from the protein-alginate complex of step (b)
2. The process as claimed in claim 1, wherein the matrix comprises 0.5 % (w/v) alginate.
3. The process as claimed in claim 1, wherein the matrix further comprises one or more composite material selected from a group consisting of chitosan, starch, cellulose, dextran, carrageenan, proteins, gelatin, oxides, SiO_2, heparin, and Hyaluronic acid.
4. The process as claimed in claim 1, wherein the protein-alginate complex of step (b) is separated by adding $CaCl_2$ solution to a final concentration of 0.06M.
5. The process as claimed in claim 1, wherein the process further comprises washing of the protein-alginate complex of step (b) by adding $CaCl_2$ solution to a final concentration of 0.06M.
6. The process as claimed in claim 1, wherein the target protein is purified by adding 0.05M to 2M Maltose solution at a temperature ranging from 4°C to 25°C for 1 to 24 hours.
7. A protein purification process comprising permitting a sample comprising a target protein to bind to a matrix comprising 0.1% to 2.0% (w/v) alginate, wherein the target protein is MBP or MBP tagged protein; and purifying the target protein from the matrix in a selected buffer to obtain purified target protein.

8. A process for producing and purifying a target protein, wherein the process comprises

    a. producing the target protein in a host cell by transforming the host cell with a recombinant vector comprising a DNA expression cassette comprising the polynucleotide encoding the target protein, wherein the target protein is MBP or MBP tagged protein,

    b. contacting the target protein with a matrix comprising 0.1% to 2.0% (w/v) alginate to obtain a protein-alginate complex,

    c. separating the protein-alginate complex, and

    d. purifying the target protein from the protein-alginate complex of step (c)

9. The process as claimed in claim 8, wherein the host cell is selected from a group consisting of a bacterial cell, a fungal cell, a mammalian cell and a plant cell.

10. The process as claimed in claim 9, wherein the bacterial cell is *E. coli*.

11. The process as claimed in any of the preceding claims, wherein the purified target protein is in the form of soluble protein or inclusion bodies.

12. The process as claimed in claim 11, wherein the inclusion bodies are solubilized in urea or guanidium chloride.

13. The process as claimed in claim 12, wherein the inclusion bodies are capable of refolding into their native conformation.

14. The process as claimed in any of the preceding claims, wherein the process is two-phase or multiphasic protein separation process.

15. The process as claimed in any of the preceding claims, wherein more than 98% target protein is purified.

16. A device for selective binding and separation of a MBP or MBP tagged target protein from a sample, wherein the device comprises housing, an inlet, an outlet

and at least one separation matrix comprising alginate, or composites of alginate.

17. The device as claimed in claim 16, wherein the alginate matrix is in form of alginate beads.

18. The device as claimed in claim 17, wherein the alginate beads are magnetic alginate beads.

19. The device as claimed in claim 16, wherein the device is packed bed column or fluidized bed column.

20. A protein purification kit comprising the device as claimed in claim 16, a precipitation and a washing buffer comprising $CaCl_2$, and an elution buffer comprising Maltose.

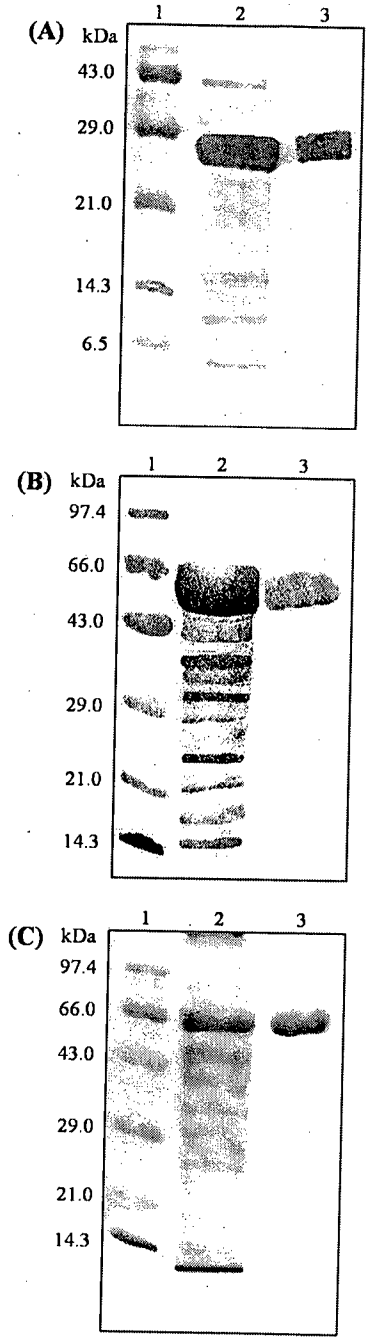

(A)
kDa
1
2
3
43.0
29.0
21.0
14.3
6.5
(B)
kDa
1
2
3
97.4
66.0
43.0
29.0
21.0
14.3
(C)
kDa
1
2
3
97.4
66.0
43.0
29.0
21.0
14.3

Figure 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2013/000092

A. CLASSIFICATION OF SUBJECT MATTER

C07K 1/14 (2006.01) C07K 1/22 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, MEDLINE, WPI, STN, BIOSIS. Keywords: maltose binding protein, MBP fusion, MBP tag, protein separation, protein purification, affinity tag, fusion protein, fusion peptide, chromatography, alginate, algin, alginic acid, calcium chloride, maltose, and similar terms, synonyms, plurals (as appropriate).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 April 2013	24 April 2013

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INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/IN2013/000092**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROY, I., and GUPTA, M., N., 'Purification of a Bacterial Pullulanase on a Fluidized Bed of Calcium Alginate Beads', Journal of Chromatography A, 2002, Vol. 950, pages 131-137. Abstract; page 134, right-hand column to page 136; Figure 4; Table 1	16-20
X	WO 2004/099777 A2 (BECTON, DICKINSON AND COMPANY) 18 November 2004 paragraphs [0007]-[0010]; [0037-0038]; Claims 5, 13, 21; Example 1, page 19	16-17
A	TEOTIA, S., and GUPTA, M. N., 'Magnetite-Alginate Beads for Purification of Some Starch Degrading Enzymes', Molecular Biotechnology, 2002, Vol. 20, No. 3, pages 231-237. page 231, left-hand column; page 232, left-hand column, paragraph 2 and right-hand column; page 234, left-hand column	1-20
A	SARDAR, M., and GUPTA, M. N., 'Alginate Beads as an Affinity Material for Alpha Amylases', Bioseparation, 1998, Vol. 7, No. 3, pages 159-165. Abstract; page 159, right-hand column, paragraph 2; page 164, left-hand column, paragraph 2; Table 1; page 161, left-hand column, paragraph 4	1-20
A	MONDAL, K., et al., 'Macroaffinity Ligand-Facilitated Three-Phase Partitioning for Purification of Glucoamylase and Pullulanase Using Alginate', Protein Expression and Purification, 2003, Vol. 28, pages 190-195. page 190, right-hand column, paragraph 3; page 192, right-hand column, paragraph 1; page 190, right-hand column, paragraph 2; page 191, right-hand column	1-20
A	MOMMA, K., et al., 'Crystal Structure of AlgQ2, a Macromolecule (Alginate)-Binding Protein of Sphingomonas sp. A1 at 2.0A Resolution', Journal of Molecular Biology, 2002, Vol. 316, pages 1051-1059. page 1051, right-hand column, paragraph 3 to page 1052 left-hand column, paragraph 1; Figure 6; page 1056	1-20

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/IN2013/000092**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2004/099777 A2	18 Nov 2004	AU 2004237079 A1	18 Nov 2004
		AU 2004237079 B2	17 Jun 2010
		CA 2524266 A1	18 Nov 2004
		EP 1620730 A2	01 Feb 2006
		JP 2006525508 A	09 Nov 2006
		NO 20055717 A	01 Feb 2006
		US 2004234962 A1	25 Nov 2004
		WO 2004099777 A2	18 Nov 2004
		ZA 200509047 A	27 Aug 2008

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.