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**Declaration under Rule 4.17:**
— *of inventorship (Rule 4.17(iv))*

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*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 2007/134365 A1

**(54) Title:** EXTRACTING AND PURIFYING β-AMYLASES

**(57) Abstract:** The invention relates to a process for the extraction and purification of β-amylase from a cell, the process comprising releasing the enzyme from the cell into a solution comprising a reducing agent and heating the cell extract in order to increase the specific activity of the β-amylase. The invention also relates to uses for an enzyme so prepared in the production or modification of foodstuffs, beverages and detergents.

# Extracting and purifying B amylases

## Field of the invention

The invention relates to extracting and purifying an enzyme from a cell, particularly, but not exclusively, to extracting and purifying a β-amylase.

## Background of the invention

β-amylase (EC 3.2.1.2), otherwise known as 1, 4-α-D-glucan maltohydrolase; saccharogen amylase; beta-amylase; or glycogenase, is an enzyme that catalyses the hydrolysis of 1, 4-α-D-glucosidic linkages in polysaccharides so as to remove successive maltose units from the non-reducing ends of the chains.

β-amylase acts on starch, glycogen and related polysaccharides and oligosaccharides producing β-maltose by an inversion. Hence β-amylase is particularly important in food industries for producing maltose.

The processes for obtaining commercial quantities of β amylase tend to be difficult to operate on a commercial scale, in terms of requiring sophisticated fermentation technology, including multiple steps for extraction, separation and purification as well as high capital investment. Some processes are characterised by an unacceptable loss in activity or wastage of β-amylase. Other processes tend to produce a crude or non purified final product that may have sub-optimal specific activity with some processes utilising Genetically Modified Organisms which require additional source identification.

In view of the above, there is a need for improved processes for the extraction and purification of β-amylase.

## Summary of the invention

The invention seeks to at least minimise one or more of the above identified problems or limitations and/or to provide an improved process for purification of β–amylase.

(a) releasing β-amylase from a cell into a solution including a reducing agent to form an extract;

(b) heating the extract to increase the specific activity of the β-amylase in the extract.

In another aspect, the invention provides β-amylase produced by the process of the invention.

In another aspect, the invention provides a cell including β-amylase produced by the process of the invention.

## Detailed description of the embodiments

As described herein, the inventor has found that the specific activity of a β-amylase in a cell extract can be increased by releasing β-amylase from a cell such as a barley cell, rootlet or grain into a solution including a reducing agent such as cysteine and heating the solution. For example, the specific activity of a heat treated extract of barley formed from a solution containing 20mM cysteine derived from milled grains was observed to increase 1.5 fold over a non heat treated sample (168.7μmoles/min/mL compared with 112.1μmoles/min/mL). Further, a heat treated extract containing 20mM cysteine was observed to have an improved specific activity of 3.5 fold (47.4μmoles/min/mL) compared with a heat treated extract without Cysteine.

In certain embodiments there is provided a process for purifying β-amylase from a cell. The process includes the steps of:

(a) releasing β-amylase from a cell into a solution including a reducing agent to form an extract;

(b) heating the extract to increase the specific activity of the β-amylase in the extract.

It will be understood that the processes of the invention are useful for purifying β–amylase from cells other than barley cells. Further, it will be understood that processes of the invention are useful for isolating barley β–amylase from cells that contain a

recombinant nucleic acid molecule that encodes barley β–amylase. Examples of such cells include bacterial cells and yeast cells.

Where the extract is formed from a barley grain, the grain is typically one which has not germinated. Such grains are useful as β-amylase tends to be accumulated during grain development and is not synthesised during germination.

Prior to forming an extract, the barley grain may be homogenised, for example by grinding in a unit such as a hammer mill. The grain may be milled using an apparatus employing counter rotating rollers. Otherwise, grains may be coarsely ground in a blender.

In certain embodiments, a cell is immersed or otherwise soaked in a solution including a reducing agent. Typically the reducing agent is cysteine. The solution typically has a concentration of cysteine ranging between about 1mM and 100 mM cysteine. Preferably the concentration of cysteine is about 20mM. However, other concentrations may be used including 5mM, 10 mM, 15mM, 30mM, 40mM 50mM, 60mM, 70mM, 80mM, 90mM, 95mM.

The solution is typically buffered to control pH. Trisma base is a suitable buffering agent. A solution having a concentration of no more than about 200mM Tris is suitable, however a solution having a concentration between about 20 to 50 mM Tris, for example, 25 mM Tris is particularly advantageous because as described herein, this concentration of Tris is compatible with, and permits separation of β-amylase on an anion exchange column.

The extract may be formed by incubation at a temperature no greater than about 10°C. These temperatures may be maintained for no more than about 3 days. For example, the extract may be maintained between 0 to about 10°C for between about 1 to 48 hours. It is particularly advantageous to maintain the extract for 4 hours at 10°C as this improves the speed of purification protocols that comprise further purification steps.

The inventor has found that an extract so formed contains various components such as enzyme inhibitors, contaminating enzymes such as phosphomonoesterase and low molecular weight protein derivatives. Further, it has been found that the specific

activity of β amylase in such an extract may be increased by heating the extract to a temperature that permits destruction of the activity of these components in the extract. As described herein, temperatures generally less than 80°C are suitable for this purpose.

It is particularly advantageous to heat the extract to between about 45 and 75°C because at temperatures approaching 70°C and above, β amylase activity may be lost. Accordingly, a temperature of about 60°C is particularly useful.

Following heating, the extract may be cooled and subjected to further purification.

The inventor has found that β-amylase can be purified to virtual homogeneity from a barley cell extract by a process including the following steps:

(a) releasing β-amylase from a cell into a solution including a reducing agent to form an extract;

(b) heating the extract to increase the specific activity of the β-amylase in the extract; and

(c) utilising ultrafiltration and ion exchange chromatography to concentrate and purify β–amylase from the heated extract.

As described herein, β-amylase can be concentrated and purified from a heat treated barley cell extract by tangential flow ultrafiltration. Accordingly, typically, in step (c), ultrafiltration is utilised to purify β-amylase from the heated extract.

In another embodiment, β-amylase can be further purified from a heat treated barley cell extract by anion exchange chromatography. Accordingly, typically, in step (c), anion exchange chromatography is utilised to purify β-amylase from the heated extract.

The process is particularly useful for modifying the oligo and polysaccharide content of an ingredient for use in the manufacture of foods and beverages. Accordingly, in one embodiment the process includes:

(a) releasing β-amylase from a cell into a solution including a reducing agent to form an extract;

(b) heating the extract to increase the specific activity of the β-amylase in the extract; and

(c) contacting the extract with a carbohydrate -containing ingredient for use in the manufacture of a food or beverage to permit β-amylase in the extract to modify carbohydrate in the carbohydrate –containing ingredient.

The carbohydrate -containing ingredient may be a dough for use in the production of breads and bread-like products, a wort for use in the production of beer and other alcohol containing beverages; a sugar mixture for use in the production of confectionary.

The process is also useful for the production of a detergent. Accordingly, in one embodiment there is provided a process for producing a detergent including:

(a) releasing β-amylase from a cell into a solution including a reducing agent to form an extract;

(b) heating the extract to increase the specific activity of the β-amylase in the extract; and

(c) providing the extract to a detergent -containing composition, to produce a detergent.

Example 1: Materials and equipment.

Germinating barley seeds (Schooner variety) were obtained from Barrett Burston Malting, (Thornleigh, NSW, Australia). Cysteine, Trisma base and hydrochloric acid were supplied by Sigma Aldrich (Castle Hill, NSW Australia). Betamyl $\beta$-amylase test reagent kit was obtained from Megazyme International Ltd (Bray, Ireland).

In one application, non germinated barley grains were coarsely ground in a Waring blender or Perten Laboratory Hammer Mill at high speed incorporating a 2mm sieve.

In another application, non germinated barley grains were immersed in Tris buffer for 2 hours and milled at 400 rpm on a Kustnel Freres & Cie roller mill at a feed rate of 2 kg per minute with a gap setting of 1mm using counter rotating smooth rollers to crack the grains allowing extraction of the enzyme.

The processed grains (100 kg) were immersed in buffer (300 Ltrs) containing 25mM Tris-HCL (pH 8.0) and 20mM cysteine and agitated in a D-Tank using counter directional paddles for 4 hours at a temperature of 10°C to extract the enzyme.

The crude β-amylase extract was then screened and centrifuged in a Sharples centrifuge at 16,000 rpm at 10°C at a feed rate of 120 Ltrs per hour to remove any precipitate.

The crude β-amylase extract was then heat processed to 65°C and recirculated for one hour in a SWEP plate heat exchanger to denature unwanted proteins before being rapidly cooled to <10°C by recirculation through a fabricated tubular heat exchanger immersed in a refrigerated glycol bath to avoid inactivation of the extracted β-amylase .

The cooled extract was then re-centrifuged at 16,000rpm at 10°C to remove flocculated proteins.

The heat treated and cooled extract was concentrated a using a Millipore prep scale CDUF006TG 6ft$^2$ polyethersulphone spiral wound cross flow ultrafiltration cartridge with 10kDa nominal molecular weight cut off run on a Masterflex economy drive peristaltic pump and Masterflex Easy load II head, at a flow rate of 400 mL per minute and 18 psi inlet pressure to separate and concentrate the β-amylase containing fractions in the retentate.

A sample of the extract was subjected to secondary purification by anion exchange chromatography using a Fast Protein Liquid Chromatography Unit (FPLC).

The buffer used for FPLC was 25mM Tris HCl (pH 8.0). The eluent buffer included 0.5M NaCl.

All solvents and buffers were degassed and filtered prior to use by passing each solution through a Millipore membrane filter, pore size 0.45 μm under vacuum for 5 minutes.

An Amersham Pharmacia AKAT gradient processing FPLC system complete with a 900 model monitor, lamp and detector (set at 280nm), 920 model pump and Frac 950 fraction collector interfaced to a Compaq Deskpro Pentium III computer supporting Unicorn analytical software was used for all protein purification. The column used for the

purification was a Hi Prep 16 /10 DEAE FF column connected to a Super loop 50 (to facilitate larger injection volumes).

Isolation of $\beta$-amylase was identified by protein bands on native electrophoresis gels and individual absorption peaks on anion exchange chromatography.

An LW Scientific UV-Vis spectrophotometer was used to measure enzyme activity operating at 410nm. The system was controlled by a Celeron processor computer supporting LW Graphite version 3.1 analytical software.

Example 2: Preparation of a Betamyl reagent substrate to calculate β amylase activity.

The analysis of $\beta$-amylase used a modified Megazyme Betamyl Method utilising a substrate containing, *p*-nitrophenyl-$\alpha$-D-maltopentose (PNPG5). Preparation involves dissolving the contents of one vial in 10mL of distilled water and using 50µL per assay with the remaining volume divided in 1mL aliquots and stored frozen between uses. The action of the Betamyl assay provides for the hydrolysis of *p*-nitrophenyl maltopentaoside to maltose and *p*-nitrophenyl maltotrioside by $\beta$-amylase, the nitrophenyl trioside is immediately cleaved to glucose and free p-nitrophenol by the $\alpha$-glucosidase present in the substrate mixture. The rate of release of *p*-nitrophenol relates directly to the rate of release of maltose by the $\beta$-amylase. The reaction is stopped by the addition of 1mL of a 1% (w/v) Trisma solution (pH 11.0) and the phenolate colour is developed by addition of Trisma base solution with absorbance read at 410nm.

One unit of enzyme activity is defined as the amount of enzyme required, in the presence of excess $\alpha$-glucosidase, required to release one micromole of *p*-nitrophenol from PNPG5 in one minute under the defined assay conditions.

Calculation of activity is defined as one $\beta$-amylase unit/g of grain = (Absorbance / Time of Assay) x (Volume in Curvette / Sample Volume) x (1 / 17.8 [Extinction coefficient]) x (Extraction Volume / Sample weight [g] x Dilution)

Example 3: Preparation of a standard curve for protein to determine β-amylase specific activity.

Protein was determined using the BioRad micro assay procedure derived from the original method of Bradford utilising a standard curve produced for bovine serum albumin. Each analysis was conducted in duplicate requiring incubation at room temperature for 10 minutes with the absorbance measured at 595 nm. Standards were prepared in the range of 0.2 to 1.4 mg/mL of protein.

Example 4: Modified Betamyl Method for the assay of $\beta$-amylase activity.

The assay requires 50µL of the extracted enzyme solution to be mixed with 50µL of the prepared Betamyl substrate reagent containing the *p*-nitrophenyl maltopentaoside and $\alpha$-glucosidase which is incubated at 40°C for 10 minutes.

The reaction is stopped with the addition of 1mL of 1% (w/v) Trisma solution (pH 11.0) and the absorbance read at 410nm. Activity is determined by calculation. A reference blank is prepared by adding 1mL of 1% (w/v) Trisma to 50µL Betamyl substrate and 50µL of distilled water.

Example 5: Preparation of crude β amylase extract.

100 kg of 3 to 12 month stored barley grains were immersed in 300 Ltrs of 25mM Tris-HCl (pH 8.0) containing 20mM Cysteine and held for 2 hours to achieve a moisture content not exceeding 40%.

The grains were then milled using counter rotating smooth rollers at a roller speed 400 rpm and gap setting of 1mm with a feed rate of 2kg of grain per minute.

Extraction of $\beta$-amylase was conducted by immersion of the milled grains in a buffered solution of 25mM Tris-HCl (pH8.0) containing 20mM Cysteine for 4 hours at 10°C to facilitate solubilization of $\beta$-amylase.

The insoluble material was removed by screening the extract through the compacted grains and a 10 micron filter. The filtrate was then centrifuged at 16,000 rpm at 10°C to remove any remaining solids and then stored at 4°C until required. This process formed the crude $\beta$-amylase extract.

The activity and specific activity of the crude $\beta$-amylase extract was determined according to Examples 2, 3 and 4 above.

Example 6: Purification of β amylase from the crude β amylase extract.

The first stage of the purification process involved the removal of any superfluous proteins from the crude β amylase extract with the aim of increasing the specific activity of the $\beta$-amylase. The crude extract was heated in a SWEP plate heat exchanger to 60°C and maintained at that temperature for 1 hour. The heated extract was then cooled in a tubular heat exchanger to <10°C and centrifuged to remove the denatured proteinaceous materials. The post heat treated extract was then concentrated and purified by cross flow ultrafiltration to facilitate down stream anion exchange chromatography. The activity and specific activity of the heat treated β amylase extract was determined according to Examples 2, 3to 4 above.

Ion exchange chromatography was undertaken by a 50mL sample of the extract injected onto a Hi Prep 16 /10 DEAE FF column connected to a Super loop 50 (to facilitate larger injection volumes) at a flow rate of 3.0 mL per minute to fractionate β amylase. The isolated fraction was removed by the elution buffer containing 0.5M NaCl and reconcentrated by ultrafiltration and stabilized in a 40% sorbitol solution. A single peak was obtained and analysed for activity and specific activity according to Examples 2, 3 and 4 above.

Example 7: Purification profile for β amylase.

The results for the purification of β amylase are shown in Table 1.

Table 1

| Sample | Activity (Betamyl Units) | Protein | Specific activity | Purification fac |
|---|---|---|---|---|
| Crude extract | 100.6 | 0.89 | 113.0 | 1.0 |
| Heat treatment | 100.5 | 0.59 | 170.3 | 1.5 |
| Ultrafiltration | 380.6 | 0.84 | 453.0 | 4.0 |
| Anion exchange | 127.3 | 0.09 | 1,414.4 | 12.5 |

Activity: μmoles/min

Specific activity: μmoles/min/mg

## Claims

1. A process for purifying β-amylase from a cell including:

(a) releasing β-amylase from a cell into a solution including a reducing agent to form an extract;

(b) heating the extract to increase the specific activity of the β-amylase in the extract.

2. The process according to claim 1 wherein the reducing agent is cysteine.

3. The process according to any one of the preceding claims wherein the solution has a concentration of cysteine from about 1mM to 100 mM cysteine.

4. The process according to any one of the preceding claims wherein the cell is a barley cell.

5. The process according to any one of the preceding claims wherein the cell contains recombinant β-amylase.

6. The process according to any one of the preceding claims wherein the cell is comprised in a grain.

7. The process according to claim 6 wherein the grain is not a germinated grain.

8. The process according to claim 6 wherein the grain is a homogenised grain.

9. The process according to any one of the preceding claims wherein the cell is immersed or otherwise soaked in the solution including a reducing agent.

10. The process according to any one of the preceding claims wherein the solution is buffered to control pH.

11. The process according to any one of the preceding claims wherein the extract is formed by incubation at a temperature no greater than about 10°C.

12. The process according to any one of the preceding claims wherein the extract is heated to a temperature of from 45 to 80°C to increase the specific activity of the β-amylase in the extract..

13. A process for purifying a β-amylase from a cell including:

(a) releasing β-amylase from a cell into a solution including a reducing agent to form an extract;

(b) heating the extract to increase the specific activity of the β-amylase in the extract; and

(c) utilising ultra filtration and ion exchange chromatography to concentrate and purify β–amylase from the heated extract.

14. The process according to claim 13 wherein the chromatography is anion exchange chromatography.

15. A β-amylase produced by a process according to any one of the preceding claims.

16. A cell including a β-amylase produced by a process according to any one of the preceding claims.

17. A process for modifying the oligo and polysaccharide content of an ingredient for use in the manufacture of foods and beverages including:

(a) releasing β-amylase from a cell into a solution including a reducing agent to form an extract;

(b) heating the extract to increase the specific activity of the β-amylase in the extract; and

(c) contacting the extract with a carbohydrate -containing ingredient for use in the manufacture of a food or beverage to permit β-amylase in the extract to modify carbohydrate in the carbohydrate –containing ingredient.

18. The process according to claim 17 wherein the ingredient is a dough for use in the production of bread, a wort for use in the production of alcoholic beverages, or a sugar mixture for use in the production of confectionary.

19. A process for producing a detergent including:

(a) releasing β-amylase from a cell into a solution including a reducing agent to form an extract;

(b) heating the extract to increase the specific activity of the β-amylase in the extract; and

(c) providing the extract to a detergent -containing composition, to produce a detergent.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/000627

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

*C12N 9/36* (2006.01) *C11D 3/386* (2006.01) *C12S 3/12* (2006.01)
*A23L 1/29* (2006.01) *C12N 9/96* (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)
MEDLINE, WPIDS, CAPLUS, BIOTECHABS (β-amylase, cysteine, reduc?)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US 4 675 296 (Lehmussaari *et al.*) 23 June 1987 (abstract; column 2, lines 18-26; examples 1 & 2) | 1-15 |
| X | US 2004/0076716 A1 (Cooper), published 22 April 2004 (abstract; [0066]-[0068]; claims 1, 11, 14, 19) | 15, 17, 18 |
| X | Derwent Abstract Accession No. 2004-456272/43, Class D16, JP 2004173552 A (Sapporo Breweries Ltd.), 26 November 2002 (abstract) | 15, 17, 18 |
| X | WO 2002/065855 A2 (Cereal Base Ceba AB), published 29 August 2002 (abstract; page 3, line 18 – page 4, line 13; claim 1) | 15, 17 |

[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 |
|---|---|
| 4 July 2007 | 11 JUL 2007 |
| Name and mailing address of the ISA/AU<br>AUSTRALIAN PATENT OFFICE<br>PO BOX 200, WODEN ACT 2606, AUSTRALIA<br>E-mail address: pct@ipaustralia.gov.au<br>Facsimile No. (02) 6285 3929 | Authorized officer<br>**JOHN SHAW**<br>AUSTRALIAN PATENT OFFICE<br>(ISO 9001 Quality Certified Service)<br>Telephone No : (02) 6283 2545 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/000627

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 1996/023873 A1 (Novo Nordisk A/S), published 8 August 1996. (page 37, lines 16-19) | 15, 19 |
| X | US 3 776 693 (Smith *et al.*), 4 December 1973. (column 2, lines 9-14) | 15, 19 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/000627

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **16**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 16 is not clear. It is directed to a cell including β-amylase produced by the process according to any one of the preceding claims, but none of the preceding claims are directed to a process of producing β-amylase, rather they are directed to processes of extracting and purifying the β-amylase naturally produced in the cells.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2007/000627

This Annex lists the known "A" publication level 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 Cited in Search Report | | Patent Family Member | | | | | |
|---|---|---|---|---|---|---|---|
| US | 4675296 | | | | | | |
| US | 2004076716 | AU | 2002349961 | CA | 2502244 | EP | 1562433 |
| | | MX | PA05004101 | US | 6896916 | US | 2004202763 |
| | | WO | 2004037003 | | | | |
| JP | 2004173552 | | | | | | |
| WO | 02065855 | AU | 10705/00 | AU | 2002258085 | CA | 2347746 |
| | | CA | 2438681 | CN | 1323167 | CN | 1491083 |
| | | EP | 1123012 | EP | 1383396 | NZ | 511439 |
| | | NZ | 527586 | RU | 2001113442 | US | 6190708 |
| | | US | 6451369 | US | 2002081367 | WO | 0022938 |
| WO | 9623873 | AR | 053946 | AU | 44833/96 | BR | 9607735 |
| | | CA | 2211405 | CN | 1172500 | CN | 1624124 |
| | | EP | 0815208 | EP | 1538155 | EP | 1752525 |
| | | US | 6093562 | US | 6297038 | US | 6867031 |
| | | US | 2002098996 | US | 2003064908 | US | 2004253676 |
| | | US | 2005059131 | US | 2005250664 | US | 2006035323 |
| US | 3776693 | | | | | | |

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

END OF ANNEX