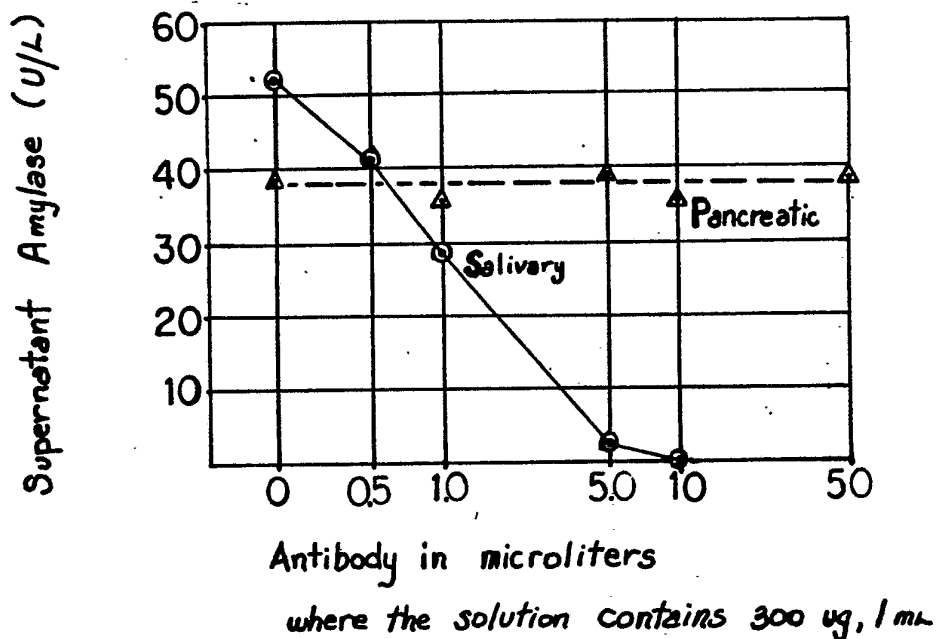




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(51) International Patent Classification⁴ : C07K 3/00, G01N 33/53 C12Q 1/42, 1/40, C12P 21/00 C12N 15/00, 5/00, 5/02 C12R 1/91, A61K 39/00 G01N 33/561, 33/543, 33/544 G01N 33/548, 33/546	A1	(11) International Publication Number: WO 87/ 00526 (43) International Publication Date: 29 January 1987 (29.01.87)
(21) International Application Number: PCT/US85/01424 (22) International Filing Date: 26 July 1985 (26.07.85) (71) Applicant: THE UNIVERSITY OF VIRGINIA ALUMNI PATENTS FOUNDATION [US/US]; Towers Office Building, Suite 6-211, 1224 West Main Street, Charlottesville, VA 22903 (US). (72) Inventors: BRUNS, David, E. ; 2516 Woodhurst Road, Charlottesville, VA 22901 (US). BENJAMIN, David, C. ; Route 2, Box 122, Earleystown, VA 22936 (US).		(74) Agent: WRAY, James, C.; 1489 Chain Bridge Road, Suite 200, McLean, VA 22101 (US). (81) Designated States: AT (European patent), BE (Euro- pean patent), CH (European patent), DE (European patent), FR (European patent), GB (European pa- tent), IT (European patent), JP, LU (European pa- tent), NL (European patent), SE (European patent). Published With international search report.

(54) Title: IMMUNOCHEMICAL ASSAYS FOR HUMAN AMYLASE ISOENZYMES AND RELATED MONOCLONAL ANTIBODIES, HYBRIDOMA CELL LINES AND PRODUCTION THEREOF



(57) Abstract

Immunochemical assays for human amylase isoenzymes. These assays utilize monoclonal antibodies which are produced from hybridoma cell lines and are specific for human salivary type amylase but not human pancreatic amylase. The assays involve reacting this monoclonal antibody with the unknown sample for the purpose of studying quantitatively and qualitatively the amylase isoenzymes present. The results from such assays are useful in the diagnosis of disease or ruling out disease. Antibody producing hybridoma cell lines and their production.

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IMMUNOCHEMICAL ASSAYS FOR HUMAN AMYLASE ISOENZYMES
AND RELATED MONOCLONAL ANTIBODIES, HYBRIDOMA
CELL LINES AND PRODUCTION THEREOF

Background of the Invention

Measurements of amylase in serum, urine, other body fluids and tissues have been used for many years as important aids in the diagnosis of a variety of conditions, especially acute pancreatitis. Amylases are usually measured in biological samples by measuring their ability to degrade starch or related compounds. Such assays measure not only amylases produced by the pancreas, but also those amylases produced by, e.g., salivary glands, fallopian tubes, etc. As a result, amylase may be increased in serum or urine when any of these other organs are diseased. This severely limits the diagnostic value of measuring amylase as an aid in the diagnosis of pancreatic disease.

There has been a longstanding need for a simple, rapid, specific method for the measurement of pancreatic amylases in biological samples. However, it has been difficult to distinguish the pancreatic and salivary-type amylases because of their close similarity. The amylases have been separated by electrophoresis, column chromatography and isoelectric focusing. However, these are, at best, slow and cumbersome. An inhibitor of salivary-type amylases has been found and applied to the measurement of pancreatic amylase. However, the inhibitor also inhibits pancreatic amylase to a lesser extent. Thus, the inhibitor method does not allow a direct measurement of pancreatic amylases. (We use the term "amylase" to indicate human alpha amylases.)

Therefore, it is an object of this invention to measure pancreatic amylases specifically in the presence of salivary and other amylases without interference from the salivary and other amylases that are closely related to salivary amylases.

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It is also a further object of this invention to do this simply, cheaply and rapidly in order that this type of test may be utilized in any routine clinical laboratory set up.

Summary of the Invention

The present inventors have achieved this invention as a result of their earnest research work to overcome such disadvantages as found with the prior art by developing the monoclonal antibody which reacts with human salivary type amylase but not human pancreatic amylase.

This invention provides a hybridoma cell line which produces a monoclonal antibody reactive with human salivary but not human pancreatic amylase.

The hybridoma is the fusion product of spleen donor cells from A/J mice, H-2^a haplotype, immunized with the purified human amylase, with SP2/0 myeloma cells H-2^d haplotype.

This invention provides an antibody which reacts with human salivary-type amylase but not with human pancreatic amylase.

The preferred antibody has structural characteristics of the IgG-2a subclass.

An embodiment of the antibody has attached to it an insoluble matrix such as latex or an immunochemical stain.

The immunochemical stain can, for example, be fluorescein or those involved in the avidin-biotin or peroxidase/antiperoxidase immunochemical staining procedures.

Still another embodiment of the antibody has attached to it alkaline phosphatase or a radioisotope where the radioisotope is ¹²⁵I or ¹³¹I.

The invention further provides a process for using a monoclonal antibody

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comprising reacting a monoclonal antibody, which is reactive with human salivary-type amylase but not with human pancreatic amylase, with an unknown sample.

One process provides taking a monoclonal antibody, which is reactive with human salivary-type amylase but not with human pancreatic amylase, which is bound to an insoluble matrix, and reacting it with an unknown sample.

The process could be any competitive protein binding procedure, such as radioimmunoassay, enzyme immunoassay or a procedure such as ELISA, i.e. enzyme linked immunosorbent assay.

Other processes to be used are double antibody techniques.

The most useful clinical method is to first determine the total amylase concentration of the unknown sample by any of the routine methods available, then taking the monoclonal antibody which is reactive with human salivary-type amylase but not with human pancreatic amylase and which has also been coupled to an insoluble matrix and reacting this with another portion of the unknown sample, centrifuging the insoluble complex down to a pellet, taking the supernatant and determining the total amylase again by any of the available methods. Total amylase in the supernatant reflects pancreatic amylase in the sample. Subtracting the last concentration determination of pancreatic amylase from the first concentration determination of total amylase results in the determination of the concentration of salivary-type amylase.

The inventors arrived at their invention by first obtaining purified, human salivary-type amylase (Zakowsky, Gregory and Bruns, Clinical Chemistry 30:62, 1984) and immunizing mice with this antigen. The spleens from these mice were excised, and a single cell suspension prepared in serum free medium.

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These cells were then mixed with SP2/0 myeloma cells at a 2:1 ratio in a centrifuge tube.

The mixture of spleen cells and myeloma cells was centrifuged. The supernatant was then drawn off, and the pellet resuspended in an aliquot of a specific type of polyethylene glycol. The polyethylene glycol promotes fusion of the cells in the mixture. The procedure results in various fusion products, which are: spleen cell-spleen cell hybrids, spleen cell-myeloma cell hybrids and myeloma cell-myeloma cell hybrids.

A selective pressure was set up which effects the isolation of the spleen cell-myeloma cell hybrids, which is the hybridoma. The hybridoma was then cloned by limiting dilution in culture. Antibody from this hybridoma has been produced in several ways: (1) harvesting supernatant from growth of the hybridoma in tissue culture; and (2) induction of an ascites tumor by injection of the hybridoma cells into the peritoneal cavity of a histocompatible CAF¹ mouse and harvesting the antibody from the resulting ascitic fluid.

Brief Description of the Drawings

Figure 1 is a graph depicting the antibody reacting with the salivary-type amylase but not the pancreatic type amylase.

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Description of the Preferred Embodiments

A monoclonal anti-amylase antibody is used to remove nonpancreatic amylases from samples such as serum, urine and tissue samples. Generally, samples containing amylases are prepared, and their total amylase content is determined by means of any suitable method. Sufficient antibody is then added to bind all of the salivary type amylase. The amylases that are bound to the antibody are then precipitated by such techniques as those using a second antibody directed against the first antibody. Alternatively, the anti-amylase activity can be coupled or bound to an insoluble support so that the bound amylases are removed from solution without the need of a second antibody, i.e., Staph A, etc.

The pancreatic amylases remaining in the samples are then measured by determining their enzymatic activity (by any convenient method) or by using an antibody-based method or by any other technique that proves capable of measuring amylases generally.

We are also able to use our antibody to measure or detect nonpancreatic amylases without interference from pancreatic amylases using such methods as immunochemical staining and/or standard immunochemical methods including radioimmunoassay (in solution or solid phase) and related immunoassays such as enzyme immunoassays and enzyme linked immunosorbent assays.

The monoclonal antibodies of the present invention were prepared by developing a hybridoma. First, various human amylases were purified, (as described in Zakowski, Gregory and Bruns, Clinical Chemistry 30:62, 1984), from saliva, pancreas and serous ovarian cyst fluid. These various human amylases were then used to immunize A/J male mice, H-2^a haplotype. Spleen

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donor cells were then taken from these mice and fused with SP2/0 myeloma cells, H-2^d haplotype. From this fusion mixture, the hybridoma was selected which produced an anti-salivary antibody which reacts also with ovarian tumor amylase and salivary amylase but not with pancreatic amylase in a standard plate-coating assay. This monoclonal antibody is of the IgG-2a subclass and was further characterized for its ability to distinguish pancreatic amylase from the other, nonpancreatic, amylases. This antibody is referred to below as the HABB antibody.

The HABB antibody was evaluated for its ability to specifically remove human salivary type amylase from solution while not affecting human pancreatic amylase. This was determined first by adding various amounts of antibody solution to 0.5 ml aliquots of either the pancreatic or the salivary amylase in pure solution. Following a 60-minute incubation at room temperature, 0.05 ml of a Staph A suspension was used to allow precipitation of the antibody bound amylases. The nonprecipitated amylase was then measured in the supernatant by measuring its enzymatic activity.

The HABB antibody, with Staph A, completely precipitated salivary amylase but did not precipitate pancreatic amylase [See Figure 1]. This same effect was obtained using HABB immobilized on other insoluble matrices, e.g. Sepharose.

To precipitate salivary amylase from serum, HABB was attached to either Staph A or Sepharose. Serum was mixed with either of these materials, and the mixture was centrifuged to remove the antibody and antibody-amylase complexes. The amylase isoenzymes in the resulting supernatant were analyzed by electrophoresis which showed that only pancreatic amylase remained.

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HABB antibody has been used to study patients with abnormally elevated amounts of amylase in serum in order to determine whether pancreatic amylase was the cause of the increased amylase. For example, one patient with an amylase-secreting tumor was thought to have pancreatitis because of a high serum amylase. Treatment of this patient's serum removed virtually all of the amylase, leading to the correct diagnosis of nonpancreatic hyperamylasemia, which was found to be caused by a gynecological malignancy.

To arrive at the HABB antibody, a purified, human salivary-type amylase had to be obtained. Several routes are available for this appropriation. There are commercially prepared purified amylases, as well as the utilization of one's own laboratory to purify such amylases. Tissue or saliva can be processed and the amylase purified by a combination of standard methods of protein purification combined with affinity chromatography. The purified amylase, then, is used to immunize A/J mice, H-2^a haplotype. The mice are then monitored and reimmunized until the desired immune response is obtained. There are a number of ways of monitoring the immune response, but, essentially, this amounts to periodic bleedings and serological testing.

Once the mice are felt to have been sufficiently immunized against the human salivary-type amylase, the spleen is then excised. The spleen contains about 10^8 lymphocytes. To these spleen cells are added 4 to 5 times 10^7 SP2/0 myeloma cells, H-2^d haplotype for an approximate 2:1 mixture. This mixture is centrifuged, and the supernatant is drawn off. The cell pellet is then resuspended with slow stirring in 1/2 ml polyethylene glycol 1000 from Kochlight Industries. The concentration of the polyethylene glycol added is 37% (v/v in serum free medium). The polyethylene glycol promotes fusion of cells resulting in a mixture of various fusion products.

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Spleen cell-spleen cell hybrids, myeloma cell-myeloma cell-spleen cell hybrids and myeloma cell-myeloma cell hybrids are obtained. The SP2/0 myeloma cell line used does not produce antibodies of its own and has certain biochemical properties which allow subsequent preferential selection of the desired hybridoma cell line. Because the different fusion products have different characteristics, selective pressure in culture can be effected to isolate the desired hybridoma. The spleen cell-spleen cell combination is not viable in vitro and thus readily dies in tissue culture. When we have eliminated the spleen cell-spleen cell combinations from the mixture, we can utilize the peculiar metabolism of the remaining fusion products to isolate the hybridoma. It is known that the myeloma cell is not able to metabolize certain precursors into essential nutrients. The hybridoma product of the fusion of the myeloma cell with the spleen cell, however, assumes the characteristic of the spleen cell and is capable of synthesizing essential nutrients and the characteristic of the myeloma cell and is capable of essentially immortal growth in culture. By using a selective medium in which to grow the fusion products, the desired hybridoma was isolated. The isolated hybridoma cells were then screened for those that produce antibody to the human salivary-type amylase. Different screening methods can be used. One particularly useful method is the enzyme-linked immunosorbent assay (ELISA assay). This is a standard serological technique using a microtiter plate. Once the desired hybridoma is isolated from the screening procedure, the cell is then cloned by limiting dilution in tissue culture. Hybridoma cells from this final clone were injected into the peritoneal cavity of CAF male mice. An ascitis tumor forms in the peritoneal cavity and the antibody is

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prepared from the resulting ascitic fluid. The antibody purified from the ascitis fluid or from supernatants of tissue culture of the hybridoma cells can be sold as is or as a complex with insoluble matrices or various enzymes, radioisotopes, immunochemical stains, etc. for use in various types of assays.

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1. A hybridoma cell line which produces a monoclonal antibody reactive with human salivary-type but not human pancreatic amylase.
2. A hybridoma cell line of claim 1 where the hybridoma is the fusion product of spleen donor cells from A/J male mice, H-2^a haplotype, immunized with the purified human amylase, with SP2/0 myeloma cells, H-2^d haplotype.
3. An antibody which reacts with human salivary-type amylase but not with human pancreatic amylase.
4. The antibody of claim 3 where the structural characteristics of the antibody are of the IgG-2a subclass.
5. The antibody of claim 3 further comprising having attached to it an insoluble matrix.
6. The antibody of claim 5 where the insoluble matrix is any suitable inert material such as latex.
7. The antibody of claim 3 further comprising having attached to it an immunochemical stain.
8. The antibody of claim 7 where the immunochemical stain is fluorescein or ones involved in the avidin biotin or peroxidase/antiperoxidase immunochemical staining procedures.
9. The antibody of claim 3 further comprising having attached to it an enzyme or other compounds attached to it, such as alkaline phosphatase, for use in assays such as the ELISA assay, fluorescence polarization, fluorescence activated cell sorting.
10. The antibody of claim 3 further comprising having attached to it a radioisotope.

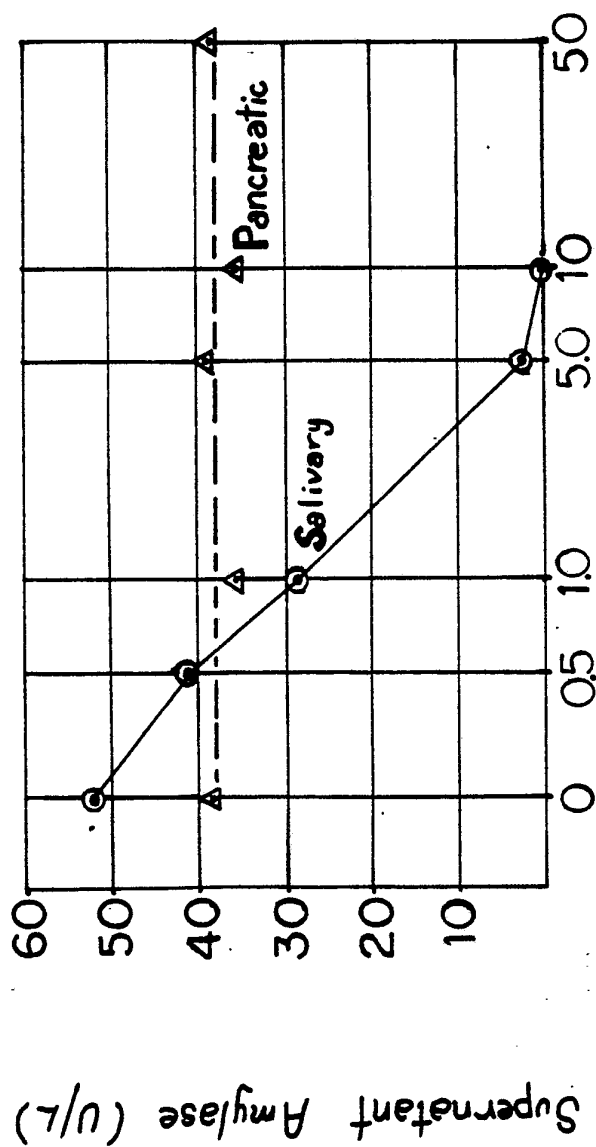
- 11 -

11. The antibody of claim 3 where the radio isotope is ¹²⁵I and ¹³¹I.
12. A process for using a monoclonal antibody comprising reacting a monoclonal antibody reactive to human salivary type amylase but not human pancreatic amylase with an unknown sample.
13. The process of claim 12 further comprising taking a monoclonal antibody reactive to human salivary type amylase but not human pancreatic amylase which is coupled to an insoluble matrix and reacting it with an unknown sample.
14. The process of claim 12 further comprising using any competitive protein binding procedure.
15. The process of claim 14 where the competitive binding procedure is radioimmunoassay.
16. The process of claim 14 where the competitive binding procedure is enzyme immunoassay.
17. The process of claim 12 further comprising using an ELISA, i.e. enzyme linked immunosorbent assay.
18. The process of claim 12 further comprising the usage of any double antibody technique or any technique used to precipitate the amylase that is bound to the HABB antibody.
19. The process of claim 12 further comprising the use of counter-immunoelectrophoresis.
20. The process of claim 12 further comprising first determining the total amylase concentration of the unknown sample by any of the routine methods available then taking that monoclonal antibody which is reactive to human salivary type amylase but not human pancreatic type amylase and which

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has also been coupled to an insoluble matrix and reacting this with an unknown sample, centrifuging the insoluble complex down to a pellet, taking the supernatant and determining the total amylase again by any of the available methods, subtracting the last concentration determination of total amylase from the first concentration determination of amylase.

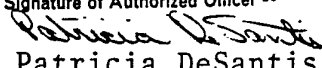
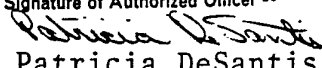
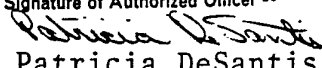
1/1



where the solution contains 300 ug, 1 ml

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/01424

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC INT. C1 ⁴ C07K 3/00, G01N 33/53, C12Q 1/42, (See Attachment) US, C1, 260/112.B; 435/7, 21, 22, 68, 172.2, 240, 241, 948; (See Att)											
II. FIELDS SEARCHED Minimum Documentation Searched ⁴ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">US, C1</td> <td style="padding: 5px;">260/112.B: 435/7, 21, 22, 68, 172.2, 240, 241, 948; 424/85; 436/516, 518, 528, 529, 533, 534, 548, 804, 813, 821; 935/89, 95, 96, 100, 103, 110</td> </tr> </table> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵			Classification System	Classification Symbols	US, C1	260/112.B: 435/7, 21, 22, 68, 172.2, 240, 241, 948; 424/85; 436/516, 518, 528, 529, 533, 534, 548, 804, 813, 821; 935/89, 95, 96, 100, 103, 110					
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category ⁸</th> <th style="border-bottom: 1px solid black;">Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷</th> <th style="width: 10%; border-bottom: 1px solid black;">Relevant to Claim No. ¹⁸</th> </tr> <tr> <td style="vertical-align: top; text-align: center; padding: 5px;">Y</td> <td style="padding: 5px;">N, Nature, Volume 256, issued 7 August 1975, G. Kohler and C. Milstein, 'Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity', pages 495-498</td> <td style="vertical-align: top; text-align: center; padding: 5px;">1-2</td> </tr> <tr> <td style="vertical-align: top; text-align: center; padding: 5px;">Y</td> <td style="padding: 5px;">N, Journal of Dental Research, Volume 63, issued 15-18 March 1984, M.I. Mednieks et al, 'Monoclonal Antibodies to α-Amylase from Rat Parotid', page 227</td> <td style="vertical-align: top; text-align: center; padding: 5px;">1-20</td> </tr> </table>			Category ⁸	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸	Y	N, Nature, Volume 256, issued 7 August 1975, G. Kohler and C. Milstein, 'Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity', pages 495-498	1-2	Y	N, Journal of Dental Research, Volume 63, issued 15-18 March 1984, M.I. Mednieks et al, 'Monoclonal Antibodies to α -Amylase from Rat Parotid', page 227	1-20
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Y	N, Journal of Dental Research, Volume 63, issued 15-18 March 1984, M.I. Mednieks et al, 'Monoclonal Antibodies to α -Amylase from Rat Parotid', page 227	1-20									
[*] Special categories of cited documents: ¹⁵ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document 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 "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											
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search ²</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report ²</td> </tr> <tr> <td style="text-align: center; padding: 5px;">9 October 85</td> <td style="text-align: center; padding: 5px;">21 OCT 1985</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">International Searching Authority ¹</td> <td style="border-bottom: 1px solid black; padding: 5px;">Signature of Authorized Officer ²⁰</td> </tr> <tr> <td style="text-align: center; padding: 5px;">ISA/US</td> <td style="text-align: center; padding: 5px;">  Patricia DeSantis </td> </tr> </table>			Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²	9 October 85	21 OCT 1985	International Searching Authority ¹	Signature of Authorized Officer ²⁰	ISA/US	 Patricia DeSantis	
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ISA/US	 Patricia DeSantis										

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
Y	N, Cinical Chemistry, Volume 30, Number 1, issued January 1984, J.J. Zakowski, et al, 'Amylase from Human Serous Ovarian Tumors: Purification and Characterization', pages 62-68	1-2
X	N, Journal of Biochemistry, Volume 97, issued May 1985, K. Ito et al, 'Preparation of Human Salivary α -Amylase Specific Monoclonal Antibody', pages 1357-1362	1-20
Y	Chemical Abstracts, Volume 96, No. 7, issued 1982, February 15, P.D. Eckersall, 'The Production and Evaluation of an Antiserum for the Detection of Human Saliva', see page 137, column 2, the Abstract No. 47068a, Journal of Forensic Sci. Soc. 1981, 21(4), 293-300 (Eng.)	3-4, 7-9, 12-13, 20

ATTACHMENT

I. CLASSIFICATION OF SUBJECT MATTER:

INT: C12Q 1/40, C12P 21/00, C12N 15/00, C12N 5/00,
C12N 5/02, C12R 1/91, A61K 39/00,
G01N 33/561, G01N 33/543, G01N 33/544, G01N 33/548,
G01N 33/546

US: 424/85;
436/516, 518, 528, 529, 533, 534, 548, 804, 813, 821;
935/89, 95, 96, 100, 103, 110