Summer 1994

Determination of Pancreatic and Salivary Amylase By Enzyme Immunoassay and Their Prevalence in Hyperamylasemic Patients

Sabdra Borgens Ward

Old Dominion University

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DETERMINATION OF PANCREATIC AND SALIVARY AMYLASE
BY ENZYME IMMUNOASSAY AND THEIR PREVALENCE
IN HYPERAMYLASEMIC PATIENTS

by
Sandra Borgens Ward
B.S. June 1968, Old Dominion College
M.S. June 1972, Old Dominion University

A Dissertation Submitted to the Faculties of
Old Dominion University and Eastern Virginia Medical School
in Partial Fulfillment of the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCE

OLD DOMINION UNIVERSITY

and

EASTERN VIRGINIA MEDICAL SCHOOL

AUGUST 1994

Approved by;

Dr. James H. Yuan, Director

Dr. Mark S. Elliot

Dr. Gerald J. Pepe

Dr. Lloyd Wolfinbarger, Jr.
ABSTRACT

DETERMINATION OF PANCREATIC AND SALIVARY AMYLASE
BY ENZYME IMMUNOASSAY AND THEIR PREVALENCE
IN HYPERAMYLASEMIC PATIENTS

Sandra Borgens Ward
Old Dominion University, 1994
Director: Dr. James H. Yuan

Currently, amylase determinations are nonspecific for the organ source and are based entirely on the enzymatic properties of amylase to produce a measurable product or byproduct. The determination of pancreatic amylase is important in the diagnosis of acute pancreatitis. Most commercially available tests for amylase employ the measurement of the change in NADH absorbance at 280 nm or of the p-nitrophenol released from a maltotetrose substrate. These are nonspecific measurements of pancreatic amylase and often necessitate other tests to be run such as a serum lipase.

The two predominant isoenzymes of amylase are pancreatic (p-amylase) and salivary (s-amylase); the most important of which is pancreatic. Pancreatic amylase to date is determined by the removal of salivary amylase by monoclonal antibody, wheat germ, or ion-exchange chromatography. Also amylase isoenzymes are determined by electrophoresis. These methods are time consuming and lack either specificity or quantification capability. There is a 7% difference in the amino acid sequences of pancreatic and salivary amylase. These structural differences between pancreatic and salivary amylase are small but significant enough that they should enable a monoclonal antibody to be produced for each isoenzyme.
A sensitive and specific microwell assay based on sandwich technique for the quantitative determination of the p-amylase and s-amylase was developed. Microwells were utilized for the solid-phase immobilization of the amylase inhibitor cycloheptaamylose (CHA) which was coupled to bovine serum albumin (BSA) by carbodiimide coupling. Monoclonal antibodies were produced against p-amylase and s-amylase (PAb and SAb). These were purified by Protein-A affinity chromatography and then coupled to horseradish peroxidase (HRPO) by carbodiimide coupling. The microwells which were coated with oxidized CHA-BSA (CHA-BSA) were incubated with patient’s serum and then allowed to react with the PAb-HRPO and SAb-HRPO.

This EIA method is able to distinguish between p-amylase and s-amylase quantitatively, rapidly and has the capability for automation. The correlation coefficients for human serum samples were 0.920 and 0.867 for p-amylase and s-amylase when comparing this EIA method and protein electrophoresis then staining for amylase. The limit of detection was determined to be 8 U/L for p-amylase and 16 U/L for s-amylase. The percent coefficient of variation for p-amylase was found to be 7.79% for the normal control and 6.28% for the abnormal control. The percent coefficient of variation for s-amylase was found to be 9.42% for the normal control and 9.29% for the abnormal control.
DEDICATION

This dissertation is dedicated to my

Parents: John and Jessie Borgens

Husband: Benjamin W. Ward

Daughters: Heather, Holly, Heidi and Hope

whose love, encouragement and support have enabled me

to pursue my education and accomplish this goal.
ACKNOWLEDGEMENT

I am indebted to Dr. James H. Yuan for his guidance, supervision and
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of this research.

I also appreciate the assistance of Sentara Norfolk General Hospital Pathology
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controls, pancreatic tissue, and amylase assays.

Last of all to Oreo, my Portuguese Water Dog, who stayed with me on nights
and weekends in the laboratory, my thanks for protection and company.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>Chapter</td>
<td></td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>A. Background</td>
<td>1</td>
</tr>
<tr>
<td>1. Chemical properties of amylase</td>
<td>1</td>
</tr>
<tr>
<td>2. Sources of amylase</td>
<td>3</td>
</tr>
<tr>
<td>3. Significance</td>
<td>4</td>
</tr>
<tr>
<td>a. Hyperamylasemia</td>
<td>4</td>
</tr>
<tr>
<td>(1) Acute pancreatitis</td>
<td>4</td>
</tr>
<tr>
<td>(2) Predisposed groups</td>
<td>7</td>
</tr>
<tr>
<td>(3) Cardiopulmonary bypass</td>
<td>7</td>
</tr>
<tr>
<td>(4) Renal insufficiency</td>
<td>9</td>
</tr>
<tr>
<td>(5) Endoscopic retrograde cholangiopancreatography</td>
<td>10</td>
</tr>
<tr>
<td>(6) Eating disorders</td>
<td>10</td>
</tr>
<tr>
<td>(7) Macroamylasemia</td>
<td>11</td>
</tr>
<tr>
<td>(8) Childhood etiologies</td>
<td>11</td>
</tr>
<tr>
<td>(9) Other</td>
<td>12</td>
</tr>
<tr>
<td>b. Hypoamylasemia</td>
<td>13</td>
</tr>
<tr>
<td>4. Test for pancreatitis</td>
<td>14</td>
</tr>
<tr>
<td>a. Amylase</td>
<td>14</td>
</tr>
<tr>
<td>(1) Total</td>
<td>14</td>
</tr>
<tr>
<td>(2) Isoenzymes</td>
<td>16</td>
</tr>
<tr>
<td>(3) Urinary</td>
<td>18</td>
</tr>
<tr>
<td>b. Other tests</td>
<td>18</td>
</tr>
<tr>
<td>(1) Lipase</td>
<td>18</td>
</tr>
<tr>
<td>(2) PLA₂</td>
<td>20</td>
</tr>
<tr>
<td>(3) Immunoreactive trypsin</td>
<td>21</td>
</tr>
<tr>
<td>(4) Acute phase proteins</td>
<td>21</td>
</tr>
<tr>
<td>(5) Miscellaneous</td>
<td>22</td>
</tr>
<tr>
<td>c. Developmental tests</td>
<td>23</td>
</tr>
<tr>
<td>(1) Radioimmunoassays</td>
<td>23</td>
</tr>
<tr>
<td>(2) Enzyme immunoassays</td>
<td>24</td>
</tr>
</tbody>
</table>

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
TABLE OF CONTENTS (Continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. Objectives</td>
<td>29</td>
</tr>
<tr>
<td>II. Experimental</td>
<td>31</td>
</tr>
<tr>
<td>A. Materials</td>
<td>31</td>
</tr>
<tr>
<td>B. Equipment</td>
<td>33</td>
</tr>
<tr>
<td>C. Methods</td>
<td>34</td>
</tr>
<tr>
<td>1. Preparation of antigens</td>
<td>34</td>
</tr>
<tr>
<td>a. Pancreatic amylase</td>
<td>34</td>
</tr>
<tr>
<td>b. Salivary amylase</td>
<td>38</td>
</tr>
<tr>
<td>2. Production of antibodies</td>
<td>39</td>
</tr>
<tr>
<td>a. Immunization of mice</td>
<td>39</td>
</tr>
<tr>
<td>b. Titer determinations</td>
<td>40</td>
</tr>
<tr>
<td>c. Cell fusions</td>
<td>42</td>
</tr>
<tr>
<td>d. Hybridoma clone selection</td>
<td>44</td>
</tr>
<tr>
<td>e. Hybridoma clone propagation</td>
<td>46</td>
</tr>
<tr>
<td>f. Hybridoma clone screening</td>
<td>47</td>
</tr>
<tr>
<td>g. Cultivation of antibodies</td>
<td>47</td>
</tr>
<tr>
<td>h. Purification of antibodies</td>
<td>48</td>
</tr>
<tr>
<td>i. Conjugation of antibodies with horseradish peroxidase</td>
<td>49</td>
</tr>
<tr>
<td>j. Isotyping of antibodies</td>
<td>50</td>
</tr>
<tr>
<td>3. Assay conditions</td>
<td>50</td>
</tr>
<tr>
<td>a. Well coating studies</td>
<td>50</td>
</tr>
<tr>
<td>b. Washing studies</td>
<td>51</td>
</tr>
<tr>
<td>c. Blocking studies</td>
<td>53</td>
</tr>
<tr>
<td>d. Sample studies</td>
<td>53</td>
</tr>
<tr>
<td>e. Antibody-HRPO studies</td>
<td>54</td>
</tr>
<tr>
<td>f. Indicator system studies</td>
<td>54</td>
</tr>
<tr>
<td>4. Study of the performance characteristics of the enzyme immunoassay</td>
<td>54</td>
</tr>
<tr>
<td>a. Linearity studies</td>
<td>54</td>
</tr>
<tr>
<td>b. Limit of detection studies</td>
<td>54</td>
</tr>
<tr>
<td>c. Precision studies</td>
<td>55</td>
</tr>
<tr>
<td>d. Cross reaction studies</td>
<td>55</td>
</tr>
</tbody>
</table>

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
# TABLE OF CONTENTS

5. Validation studies of human sera ............................................. 55  
   a. EIA ........................................................................... 55  
   b. Total amylase ............................................................. 56  
   c. Electrophoresis ......................................................... 56

III. RESULTS ................................................................................................57  

A. Preparation of antigens ............................................................... 57

B. Production of antibodies ............................................................... 57  
   1. Immunization of mice ............................................................. 57  
   2. Titer determinations ............................................................. 59  
   3. Cell fusions ........................................................................... 59  
   4. Production of antibodies in vitro .............................................. 63  
   5. Production of antibodies in ascites fluid .................................... 63  
   6. Purification of antibodies ......................................................... 65  
   7. Isotyping of antibodies .............................................................. 65

C. Assay conditions .............................................................................. 65

   1. Well coating studies ............................................................. 65  
   2. Washing studies ................................................................. 71  
   3. Blocking studies ................................................................. 75  
   4. Sample studies ................................................................. 75  
   5. Antibody-HRPO studies ....................................................... 82  
   6. Indicator system studies ....................................................... 91

D. Study of the performance characteristics of the enzyme  
   immunoassay ........................................................................... 91

   1. Linearity studies ................................................................. 91  
   2. Limit of detection studies ..................................................... 91  
   3. Precision studies ............................................................... 97  
   4. Cross reactivity studies ....................................................... 97

E. Validation studies ............................................................................. 97
TABLE OF CONTENTS (Continued)

IV. DISCUSSION .................................................................................... 104
   A. Amylase .......................................................................................... 104
       1. Purification .............................................................................. 104
       2. Amylases standards ............................................................... 104
       3. Chemical properties ............................................................... 105
   B. Cycloheptaamylose ..................................................................... 106
   C. Antibody production ................................................................. 109
       1. Cell fusion .............................................................................. 109
       2. Ascites fluid production .......................................................... 110
   D. Diagnostic groups ....................................................................... 111
   E. Human serum samples .............................................................. 112
       1. Sample diluents .................................................................... 112
       2. Enzyme immunoassay ............................................................. 113
       3. Electrophoresis ...................................................................... 114
   F. Summary ..................................................................................... 115

BIBLIOGRAPHY ................................................................................. 119

APPENDIX ............................................................................................ 132
### LISTS OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Summary of pancreatic amylase extraction and purification</td>
<td>58</td>
</tr>
<tr>
<td>2. Summary of cell fusions</td>
<td>60</td>
</tr>
<tr>
<td>3. Summary of antibody production</td>
<td>64</td>
</tr>
<tr>
<td>4. Summary of the assay conditions for enzyme immunoassay for p-amylase and s-amylase determination</td>
<td>94</td>
</tr>
<tr>
<td>5. Precision studies of p-amylase determination</td>
<td>98</td>
</tr>
<tr>
<td>6. Precision studies of s-amylase determination</td>
<td>99</td>
</tr>
<tr>
<td>7. Comparison of electrophoresis and enzyme immunoassay of p-amylase and s-amylase on human serum samples</td>
<td>101</td>
</tr>
</tbody>
</table>
# FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Principle of the homogeneous enzyme immunoassay</td>
<td>26</td>
</tr>
<tr>
<td>2. Principle of the competitive heterogeneous enzyme immunoassay</td>
<td>27</td>
</tr>
<tr>
<td>3. Principle of the sandwich heterogeneous enzyme immunoassay</td>
<td>28</td>
</tr>
<tr>
<td>4. Activation of Sepharose with 1,4-butanediol diglycidal ether</td>
<td>36</td>
</tr>
<tr>
<td>5. Reaction of coupling CHA to Sepharose</td>
<td>37</td>
</tr>
<tr>
<td>6. Cell fusion schematic</td>
<td>45</td>
</tr>
<tr>
<td>7. Reaction of coupling CHA to BSA</td>
<td>52</td>
</tr>
<tr>
<td>8. Titer assay of anti-p-amylase by solid-phase enzyme immunoassay on microwell immobilization</td>
<td>61</td>
</tr>
<tr>
<td>9. Titer assay of anti-s-amylase by solid-phase enzyme immunoassay on microwell immobilization</td>
<td>62</td>
</tr>
<tr>
<td>10. The elution profile of anti-p-amylase from protein A Sepharose chromatography</td>
<td>66</td>
</tr>
<tr>
<td>11. The elution profile of anti-s-amylase from protein A Sepharose chromatography</td>
<td>67</td>
</tr>
<tr>
<td>12. Structure of cycloheptaamylose</td>
<td>68</td>
</tr>
<tr>
<td>13. Infrared analysis of the oxidation of cycloheptaamylose at 0, 8, 12, and 24 hours</td>
<td>69</td>
</tr>
<tr>
<td>14. Infrared analysis of the oxidation of cycloheptaamylose at 36, 42, 48, and 84 hours</td>
<td>70</td>
</tr>
<tr>
<td>15. Optimization of coupling oxidized cycloheptaamylose to bovine serum albumin with 1 mM aqueous 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide for assay in solid-phase enzyme immunoassay on microwell immobilization</td>
<td>72</td>
</tr>
</tbody>
</table>
### List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.</td>
<td>Optimization of coupling oxidized cycloheptaamylose to bovine serum albumin with 0.1 M aqueous 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide for assay in solid-phase enzyme immunoassay on microwell immobilization</td>
</tr>
<tr>
<td>17.</td>
<td>Washing study on solid-phase enzyme immunoassay on microwell immobilization</td>
</tr>
<tr>
<td>18.</td>
<td>Study of dilutions of blocking agents on solid-phase enzyme immunoassay on microwell immobilization</td>
</tr>
<tr>
<td>19.</td>
<td>Blocking incubation study of 0.5% bovine serum albumin at room temperature on solid-phase enzyme immunoassay on microwell immobilization</td>
</tr>
<tr>
<td>20.</td>
<td>Blocking incubation study of 0.5% bovine serum albumin at 37°C on solid-phase enzyme immunoassay on microwell immobilization</td>
</tr>
<tr>
<td>21.</td>
<td>Blocking incubation study of 3% bovine serum albumin at room temperature on solid-phase enzyme immunoassay on microwell immobilization</td>
</tr>
<tr>
<td>22.</td>
<td>Blocking incubation study of 3% bovine serum albumin at 37°C on solid-phase enzyme immunoassay on microwell immobilization</td>
</tr>
<tr>
<td>23.</td>
<td>Sample diluent study on solid-phase enzyme immunoassay on microwell</td>
</tr>
<tr>
<td>24.</td>
<td>Sample size study on solid-phase enzyme immunoassay on microwell</td>
</tr>
<tr>
<td>25.</td>
<td>Sample incubation study at room temperature on solid-phase enzyme immunoassay on microwell immobilization</td>
</tr>
<tr>
<td>26.</td>
<td>Sample incubation study at 37°C on solid-phase enzyme immunoassay on microwell immobilization</td>
</tr>
<tr>
<td>27.</td>
<td>Antibody dilution study of cross reacting anti-p,s-amylase-HRPO (2PC12) on solid-phase enzyme immunoassay on microwell immobilization</td>
</tr>
<tr>
<td>28.</td>
<td>Antibody dilution study of anti-p-amylase-HRPO (2PC2) on solid-phase enzyme immunoassay on microwell immobilization</td>
</tr>
</tbody>
</table>
List of Figures..........................(Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>29. Antibody dilution study of anti-s-amylase-HRPO (2S2F7) on solid-phase enzyme immunoassay on microwell immobilization</td>
<td>88</td>
</tr>
<tr>
<td>30. Antibody incubation study at room temperature on solid-phase enzyme immunoassay on microwell immobilization</td>
<td>89</td>
</tr>
<tr>
<td>31. Antibody incubation study of antibody at 37°C on solid-phase enzyme immunoassay on microwell immobilization</td>
<td>90</td>
</tr>
<tr>
<td>32. Color development time study of p-amylase solid-phase enzyme immunoassay on microwell immobilization</td>
<td>92</td>
</tr>
<tr>
<td>33. Color development time study of s-amylase solid-phase enzyme immunoassay on microwell immobilization</td>
<td>93</td>
</tr>
<tr>
<td>34. Linearity of anti-p-amylase-HRPO (2PC2) on solid-phase enzyme immunoassay on microwell immobilization</td>
<td>95</td>
</tr>
<tr>
<td>35. Linearity of anti-s-amylase-HRPO (2S2F7) on solid-phase enzyme immunoassay on microwell immobilization</td>
<td>96</td>
</tr>
<tr>
<td>36. Principle of the enzyme immunoassay for p-amylase or s-amylase</td>
<td>107</td>
</tr>
<tr>
<td>37. Electrophoresis of human serum samples</td>
<td>116</td>
</tr>
</tbody>
</table>
Chapter I

INTRODUCTION

A. Background

1. Chemical properties amylase

Pancreatic α amylase (α-1,4-glucan 4-glucanohydrolase EC 3.2.1.1) is present in two equally active forms (I and II). To date, structural studies have been performed on porcine pancreatic amylase. Its molecular weight has been calculated to be 54,967.\textsuperscript{1,2} It is also not significantly glycosylated.\textsuperscript{1} Porcine pancreatic α-amylase consists of three domains. The N-terminus contains: domain A, the largest portion, and domain B. Domain A folds into a typical (α/β)\textsubscript{8} barrel structure. Between the third beta-strand and the third helix of domain A lies domain B. This region has two antiparallel beta-sheets and a long loop, whose internal structure is not as regular. Domain B is involved in substrate binding.\textsuperscript{3} Binding studies indicate that there are two binding sites: one in a deep cleft that exists 3 nm from the carboxyl end of the alpha/beta barrel, which is believed to be the active site; the other binding site is believed to be on the surface of the molecule about 2 nm away from the active site region.\textsuperscript{4} A globular C domain exists in the C-terminal region. It consists of a chain folded into an 8-stranded antiparallel beta-barrel.

A calcium ion appears to form an ionic bridge between domain A and B.\textsuperscript{3} The attachment of the calcium ion is essential for the structural integrity of amylase. Calcium ions can be replaced by strontium or barium ions. Chloride ions are known to be required for amylase activity.\textsuperscript{4,5} Chloride’s attachment is at the center of the

1

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alpha/beta barrel and is bound to a specific lys residue. Chloride can be replaced by a bromide ion.  

Robyt and French studied the binding sites of maltotriose (G3) through maltoctaose (G8) with the use of $^{14}$C label in the reducing glucose unit. Maltodextrin G3 was cleaved at bond one and two from the reducing end with the highest frequency at bond one. As the length increased to G4, bonds one and two were still cleaved; however, the highest frequency became bond two. Cleavage of maltodextrins G5 through G7 remained at bond 2; however, the maximum cleavage shifted with G8 to bond 3.

The active binding site was therefore proposed to bind a maximum of five glucose units. Also, the binding of the substrate appeared to be polar with the reducing end to the right of the catalytic groups. With G5 and higher oligosaccharides, the cleavage was explained by the tendency of the molecule to occupy as fully as possible the entire binding site; thus, the ends overlap, and cleavage was at the number three bond. This was supported by the fact that G5 was resistant to cleavage of all bonds except number two.

As the concentration of the substrates increased, the products shifted. The data supported the fact that at low concentrations of G3 the products are due to hydrolysis since G1 was produced at a faster rate than G2. At high concentrations, G2 was formed at a faster rate. This supports the hypothesis that the products are the result of condensation of two G3 molecules to produce a G6, which subsequently undergoes hydrolysis to give three G2's.
Maltodextrin G4 demonstrates a concentration dependence of the product distribution; however, the results are not as easily interpreted. The most reasonable explanation was still that a condensation reaction takes place initially, and then hydrolysis takes place. The frequency of cleavage suggested that the most stable binding favors formation with cleavage at bond three.  

There is a 7% difference in the amino acid sequences of the two predominant isoenzymes, salivary (s-amylase) and pancreatic amylase (p-amylase). They also differ in their activation energies and enthalpy changes. Electrophoresis yields the following isoenzymes of amylase: two pancreatic forms (P₁ and P₂) and sometimes a third (P₃), and two salivary forms (S₁ and S₂). P₃ is not found in normal pancreatic tissue but does occur in acute pancreatitis. The most common isoforms found are P₂ and S₁. On migration on cellulose acetate and agarose S₂ migrates closest to the anode, followed by S₁, P₃ if present, P₂ then P₁.  

2. Sources of amylase  

Human amylase is found in a variety of sources: pancreas, saliva, serum, urine, tears, breast milk, sweat, amniotic fluid, ovary, fallopian tubes and testes. The two most concentrated areas of amylase are the salivary glands and the pancreas with the following values: parotid 1710 ± 897 U/g, submandibular 605 ± 354 U/g, and pancreas (258 ± 137 U/g). Normal pancreatic tissue contains no s-amylase. Normal total serum amylase is 25-125 U/L.  

Compared to the pancreas other tissues of the digestive tract contain 1/35 to 1/45 the amount of amylase. The amylase that is present in these tissues is composed
of 25% p-amylase and 75% s-amylase. Values of other tissues contain 1/100 to 1/1000 less amylase as compared to the salivary glands and pancreas. Tissues that contain more than 90% p-amylase are the pancreas, jejunum, liver, placenta, testis, skeletal muscle, and spleen. Only the salivary and thyroid contained more than 90% of the s-amylase. All the other tissues contain mixtures of the two isoenzymes.11

Amylase is eliminated from the body via the glomerulus of the kidney and is not reabsorbed by the tubules.16 Therefore, urinary amylase levels reflect the serum level since the enzyme is not usually reabsorbed by the tubule except in renal tubular damage. Toxic compounds like the proteolytic enzymes of pancreatitis and ethanol can effect not only the pancreas but the renal tubules as well resulting in an elevated amylase.17

3. Significance

a. Hyperamylasemia

(1) Acute pancreatitis

At one time hyperamylasemia was believed to be an exclusive indicator of pancreatic or salivary disease.18 The determination of p-amylase is still important in the diagnosis of acute pancreatitis.19 Causes of acute pancreatitis include obstruction, vascular disorders, trauma, toxins and a variety of metabolic disorders. The one common factor of all forms of acute pancreatitis is the increased permeability of the cell membrane and the autodigestion of the pancreatic proteolytic enzymes.20 Increase of serum amylase is the result of an increased rate of entry into the circulation and/or the decreased clearance of this enzyme. Although the pancreas and salivary glands are
the two sources with the greatest amount of amylase,\textsuperscript{14} conditions other than pancreatitis and salivary disease have now been attributed to hyperamylasemia: such as, tumors of the lung, ovary,\textsuperscript{12} and breast;\textsuperscript{21} intestinal obstruction or inflammation; common-duct stones; hepatitis; facial or abdominal trauma\textsuperscript{22}; autoimmune diseases;\textsuperscript{23} chronic alcoholism; postoperative cardiopulmonary bypass; eating disorders; lactic acidosis; renal failure; macroamylasemia; and total body irradiation.\textsuperscript{24}

In a comprehensive study of 368 patients over a 4 year period, the incidence of acute pancreatitis revealed: 56\% related to gallstones, 16\% to alcohol, and 28\% idiopathic.\textsuperscript{25} A similar study showed slightly different percentages: 55\% alcohol abuse, 27\% gallstones, 6\% pancreatic cancer, 12\% miscellaneous.\textsuperscript{26} One study in Scotland showed the most common etiology of pancreatitis to have been biliary tract disease (30\% of the males; 53\% of the females). Pancreatitis related to alcohol occurred in 27\% of the males, but in only 3\% of the females. The most common complication was pancreatic pseudocysts, then pancreatic abscesses, renal failure, respiratory failure, and last disseminated intravascular coagulation.\textsuperscript{27}

Sources of hyperamylasemia in a study by Dougherty showed 32\% was due to gallstones, 49\% alcoholic patients, and 19\% other. In the gallstone patient group their amylase levels were significantly higher than the non gallstone groups; also the serum amylase decreased significantly in 24 hours. On surgery no or little pancreatitis was found in the gallstone group.\textsuperscript{28}

Certainly the two most common groups are biliary or alcohol related. The migration of a gallstone causing the blocking of the ampulla of Vater can cause a
gallstone pancreatitis.²⁹ In some individuals, the ampulla of Vater is shared with the bile duct. In others it is separate but lies within close proximity.³⁰ This diagnosis can be made based on history, physical exam and a serum amylase above 1000 IU/L, ultrasound, and CT scans.²⁹

With the high correlation to alcohol abuse one would think that the reciprocal would be true: individuals with alcohol abuse have a higher incidence of pancreatic disease than the normal population. However, in a study of individuals arrested for drunk driving, it was found that pancreatic enzyme levels were twice normal in only 3/300; whereas, hepatic enzymes were twice normal in 31/300. It was concluded that there were other factors besides alcohol that predisposed persons to acute pancreatitis and that the liver is more sensitive to alcohol abuse than the pancreas.³⁰

Pancreatitis first presents itself with the following symptoms (in order of their prevalence): epigastric pain, localized or generalized tenderness, nausea and vomiting, fever, palpable mass and ascites.³¹ Symptoms are similar with the most common complaint being abdominal pain.³²,³³ Most helpful in diagnosis was hyperamylasemia; then elevated amylase/creatinine clearance ratio (Cₐ₉/ₐ₉).³⁴ Lipase values are also often included.³⁵ Autopsies over a five year period showed that out of 43 patients having acute pancreatitis, the disease was first diagnosed at autopsy in 30% of the cases. The diagnosis could have been present on admission in over half of these. Alcoholism, biliary tract disease, and surgery around the pancreatic area were among the most common causes. Abdominal pain was present in only one patient. Amylase levels were determined in 25% of the cases, but only 10% had amylase values in the
diagnostic range (equal or greater than three time the upper limit. Wilson and Imrie raised the question why the diagnosis of acute pancreatitis was missed so frequently. In their review of deaths due to acute pancreatitis over a 10 year period, acute pancreatitis was not diagnosed until autopsy in 33% of the cases. Only 7% had abdominal pain. The majority, 68%, presented an atypical picture with another known or suspected condition or were a postoperative result. Amylase determinations were made in only 9% of the cases. They concluded that non-diagnosis was most often due to the lack of considering acute pancreatitis as a cause; hence, no diagnostic test that would reveal acute pancreatitis was performed.

(2) Predisposed groups

Some groups of individuals have been identified to be predisposed to hyperamylasemia and/or pancreatitis. In homozygous sickle cell patients, it was found that 50% had elevated serum amylase values. This seems to suggest that they may be predisposed to chronic pancreatitis.

Studies by Prinz have led to the conclusion that whereas infarction can induce inflammation and necrosis of the pancreas, ischemia may aggravate but does not cause pancreatitis. Also in a study of 52 patients with postoperative pancreatitis, 30 had previous procedures near the pancreas. The most common procedures were: biliary, colectomy and intestinal resection.

(3) Cardiopulmonary bypass

Of particular note is the high incidence of hyperamylasemia and/or pancreatitis related to cardiopulmonary bypass surgery. In a study of three hundred patients
undergoing cardiopulmonary bypass, 32% developed hyperamylasemia. Within this group 58% were asymptomatic and had normal lipase, 33% had subclinical pancreatitis, and 8% actually had acute pancreatitis. Isoamylase studies indicated that the hyperamylasemia was not due to p-amylase. Mortality was higher in patients with hyperamylasemia.\textsuperscript{41} A study by Chang of 75 patients had similar results. Hyperamylasemia was seen in 36% of the patients. Of these patients, 3 out of the 27, developed acute pancreatitis. Analysis of these patients revealed that coronary artery disease predisposed a patient to post bypass hyperamylasemia. This phenomena has to be closely monitored since there is such a high incidence of non pancreatic hyperamylasemia, and yet the possibility of acute pancreatitis existed.\textsuperscript{42} Some reports suggest ischemia may be the cause of a greater risk of developing pancreatitis after undergoing cardiopulmonary bypass, but this has not been proven.\textsuperscript{43}

Evidence that associates the preoperative administration of calcium chloride in cardiac surgery to have a high correlation with hyperamylasemia has also been implicated. Fernandez analyzed 300 patients undergoing cardiopulmonary bypass and reported "pancreatic cellular injury was significantly associated with preoperative renal insufficiency, valve surgery, postoperative hypotension, and preoperative administration of calcium chloride."\textsuperscript{43} The risk of pancreatic cellular injury was also determined to be dose related to the calcium chloride. Administration of more than 800 mg of calcium chloride per square meter of body-surface area appeared to be a predictor and possibly the cause of pancreatic cellular injury.\textsuperscript{43}
In cardiac surgery, hyperamylasemia was seen in 36% of patients with bypass surgery, 59% of patients with valve replacement, and 69% undergoing both procedures. Lipase was increased in 30% of the cardiac surgeries. It was found that 36% of the patients had an increase in s-amylase, which also correlated with the severity of pleural effusion. This may have been due to the absorbance of s-amylase from pleural fluid and/or from aspirated salivary secretions.44

In children with open heart surgery, it was found in a five year study that 54 patients had elevated amylase values or clinical pancreatitis following surgery. Of these 33 had elevated amylase values only. The remaining exhibited pancreatitis along with elevated amylase values. The etiology was proposed to be vascular in origin.45

(4) Renal insufficiency

Another cause of hyperamylasemia is abnormal renal function. In 1987, Bardella reported that 33 out of 37 chronic renal failure patients had elevated amylase levels, which correlated to the duration of the chronic renal failure. Pancreatic amylase, however, was only slightly elevated in three of these patients. Blood urea nitrogen (BUN) and serum creatinine showed no correlation to serum amylase.46

Values of amylase, along with lipase and trypsin, can become elevated when the creatinine clearance is less than 50 mL/min.47 In chronic renal failure both the amylase values and the C_{mm}/C_{cr} are increased; however, the p-amylase to s-amylase ratio is normal.48 These elevated amylase and lipase values have been reported in chronic renal failure patients in the absence of clinical pancreatitis. However, amylase values of greater than threefold in peritoneal fluid may be indicative of pancreatitis.49
Interestingly, the total amylase was found to be significantly elevated in 34 end-stage renal disease and was not reduced after hemodialysis. However, serum lipase, which was likewise increased before dialysis, increased further after dialysis. The increase in lipase correlated with the cumulative doses of heparin. P3 isoamylase was normal in all patients.50

(5) Endoscopic retrograde cholangiopancreatography

Endoscopic retrograde pancreatography (ERCP) is most helpful in determining if blunt trauma injury to the pancreas has caused any ductal injury.51 However, this procedure is not without its inherent problems. The first is that ERCP has been shown to elevate all of the major pancreatic enzymes (serum amylase; lipase; trypsin; elastase; serum alpha 1-antitrypsin and alpha 2-macroglobulin, two major pancreatic protease inhibitors) in a study of 25 patients undergoing this procedure. The second is that in three patients pancreatitis was induced by the ERCP.52 This ERCP induced pancreatitis is not that uncommon. In another study the incidence was even higher. In the follow up of 31 patients undergoing ERCP, 11 developed pancreatitis. The incidence was the highest in which maneuvers, such as stent placement and balloon dilation, were performed.53

(6) Eating disorders

In eating disorders, such as anorexia nervosa and bulimia, amylase levels as high as 3000 U/L have been seen; however, p-amylase, lipase, and trypsinogen were all normal.54 A study of 17 patients with eating disorders revealed that out of the six
patients with hyperamylasemia, five had increased s-amylase while all had normal lipase and p-amylase.55

(7) Macroamylasemia

The first reported case of hyperamylasemia due to the formation of an amylase-globulin complex was by Wilding in 1964.56 The term "macroamylase" was first used by Berk in 1967 to describe persistently elevated serum amylase without hyperamylasuria.56,57 Early sources indicated a preponderance of the s-amylase coupled to the antibody;12 however, more recent sources seem to indicate that the amylase in macroamylase has an equal distribution of and p-amylase.58 The antibody involved in macroamylase formation appears to be a monoclonal antibody.58 It has been reported to be IgA, IgG or in some instances a polysaccharide(s) or glycoprotein(s).59 The abnormally large complex, 150,000 to 2,000,000 Da,60 is unable to be excreted in the urine even when there is no renal dysfunction. This results in an elevated serum amylase levels.60,61 It is clinically important since it appears to indicate a pancreatitis when there is none.

(8) Childhood etiologies

Etiologies of childhood pancreatitis are different than those of adults. Ziegler stated that the most common causes of this disease in children were biliary tract disease, trauma and congenital abnormalities, which frequently required surgery. Biliary tract disease accounted for 33% of the pancreatitis cases, half of these were related to a hematological disease, usually sickle cell. Of equal cause (33%) was trauma with one third the result of child abuse. Other causes of childhood pancreatitis
were: systemic disease, congenital abnormalities, and idiopathic. Other studies showed similar etiologies to adults along with the category of drug induced pancreatitis. However, there is disagreement as to the prevalence of various causes. In Tagge’s study over an eleven year period involving 33 children, pancreatic causes were diagnosed as: ductal abnormalities being the most common, with 12 cases; trauma, 10; idiopathic, 4; gallstones, 3; drug induced, 3; and tumor, 1.35

A study of 61 children with acute pancreatitis showed that one third of the cases were due to multisystem disease (Reye syndrome, sepsis, shock, hemolytic-uremic syndrome, viral infections). Also as causes were blunt trauma, structural defects, metabolic disease and drug toxicity, and idiopathic.33

A study over a 28 year period involving 48 children with pancreatitis showed the following etiologies: 16 were idiopathic; 12 were drug induced, all of which were the result of corticosteroid use; 9 were the result of blunt trauma; 7 had obstruction of the pancreaticobiliary duct system; two were associated with sepsis; and two had recurrent hereditary pancreatitis.34

(9) Other

Certain drugs are well known to elevate serum amylase levels. Morphine raises amylase values up to three times normal because opiates constrict the sphincter of Oddi, which causes back pressure into the pancreatic duct. Other drugs known to cause an increase in amylase levels are choline, methacholine, chlorothiazide, pancreozymin, and secretin.20
Total body irradiation has resulted in increased serum amylase, most of which was due to s-amylase, with only 27% due to p-amylase.\(^2\)

Of a more unusual nature vitamin D intoxication has been validated as a cause of recurrent pancreatitis. A patient was admitted four times with acute pancreatitis and had serum calcium levels between 13.5 and 14.5 mg/dL. Questioning of the patient revealed the intake of excessive quantities of vitamin D. When the vitamin D intake ceased, the pancreatitis did not reoccur.\(^6\)

There still remain some cases of hyperamylasemia that cannot be explained. Warshaw investigated 117 patients whose amylase values remained elevated from three to forty-eight weeks. Extensive investigation failed to show any reason for the persistent elevation. Non pancreatic causes of their hyperamylasemia were attributed to 79% of the cases as ascertained by polyacrylamide gel electrophoresis. Macroamylasemia was the cause of 6%. Normal distribution of the isoenzymes were found in 64% of the patients although they were high. Salivary amylase was elevated in 9\(^6\)^{3}

b. Hypoamylasemia

Normal amylase values can occur even in patients with acute pancreatitis. They can be related to 1) prevalence of alcoholic etiology, 2) a number of previous alcoholic pancreatitis incidents, and/or 3) a duration of symptoms greater than two days. Lipase values were elevated in 68% of this group. Acute pancreatitis in patients with normal amylasemia is treated the same as those with an elevated amylase levels.\(^6\) There has also been reported acute pancreatitis with low to normal amylase
values when there is a pancreatic isoamylase deficiency. The acute pancreatitis was confirmed surgically; however, the trypsin was elevated. Also low p-amylase values have been reported in chronic exocrine pancreatic disease, such as chronic pancreatitis, carcinoma of the pancreas, cystic fibrosis, and of rarer occurrence, a congenital deficiency.

4. Test for pancreatitis

a. Amylase

(1) Total

There are three main methods for determining amylase activity. First, amylolastic, in which the free substrate, usually starch, is measured. Second, saccharogenic, in which the products (reducing sugars), are measured. Last chromolytic, in which the substrate is composed of amylose or amylopectin conjugated to a dye at C-2, and the resulting water soluble product (dye-glucoside) is measured. Currently most commercially used tests for amylase employ the measurement of the change in NADH absorbance at 280 nm or of the p-nitrophenol released from a maltotetrose substrate. Other substrates that have been investigated include: ethylidene-blocked 4-nitrophenyl-maltoheptaoside (EPS), where the absorbance of 4-nitrophenol that is released is measured. Also, o-(4,6-o-isopropylidene-α-D-glucopyranosyl)-(1→4)-[o-α- D-glucopyranosyl-(1→4)]-5-o-α-D-glucopyranosyl-(1→2)-α-D-fructofuranoside (IPG7F) has been used with the subsequent determination of the oxidation rate of NADH. Likewise, 2-chloro-4-nitrophenyl-α-maltotrioside can be used as a substrate, with the release and
measurement of 2-chloro-4-nitrophenol. These only determine total amylase, i.e. they are nonspecific for p-amylase and often necessitate other tests to be performed such as serum lipase.

Amylase has come under much scrutiny as to being the best indicator of pancreatic disease. The usual indications for acute pancreatitis are abdominal pain and a serum amylase levels three times the upper normal limit. Levels less than this value are usually due to conditions other than acute pancreatitis. Lin suggested from his studies that amylase values were diagnostic if the cutoff was three or four times the upper limit, and at this level amylase determination was similar in sensitivity and specificity to p-amylase and lipase determinations. Diagnostic accuracy of serum amylase, p-amylase, lipase, trypsinogen and elastase-1 were: 96%, 96%, 93%, 91%, and 84% respectively in 100 patients admitted as emergencies to the surgical department and 27 proven acute pancreatitis patients in the study. Gwozdz likewise found that on hospital admission all serum assays were equally sensitive; however, on later tests, lipase, trypsinogen and elastase-1 were more sensitive than serum amylase. The C_m/C_C was found not to be differential.

There appears to be a growing consensus that amylase values are not as elevated in alcoholic causes as in other causes, particularly in biliary tract disease, and that amylase values decrease more rapidly in time than some of the other serum enzyme tests. The sensitivity of serum amylase, using greater than 1000 U/L, was evaluated in 417 patients with acute pancreatitis; 62% were a result of gall stones, 25% from alcohol, 13% of unknown cause. On admission amylase was diagnostic,
using greater than 1000 U/L, in identifying 96% of the mild cases and 87% of the severe. At 48 hrs this was reduced to 33% and 48%, respectively. Hiatt found in his study that hyperamylasemia due to biliary tract disease was higher, decreased more rapidly and to a lower value than hyperamylasemia due to alcoholic pancreatitis. The occurrence of normal amylase levels in acute pancreatitis can be related to 1) prevalence of alcoholic etiology, 2) a number of previous alcoholic pancreatitis incidents, and/or 3) a duration of symptoms greater than two days. Lipase values were elevated in 68% of this group. Analysis of peritoneal fluid did not increase the diagnostic sensitivity. However, bloody ascites and elevated amylase was helpful in determining the presence of necrosis and hemorrhage.

There does not appear to be a correlation between amylase levels and pancreatic involvement as seen by computed tomography.

(2) Isoenzymes

Pancreatic and salivary amylase can be separated by the removal of s-amylase by a monoclonal antibody; wheat germ, which does not completely inhibit salivary and does inhibit pancreatic to some extent; ion-exchange chromatography; or electrophoresis. These methods are time consuming and lack either specificity or quantification. However, the need to differentiate p-amylase from s-amylase remains, and the presence of the P3 isoenzyme appears to be significant in the diagnosis of acute pancreatitis.

Pancreatic amylase appears to be more sensitive than total amylase in the diagnosis of acute pancreatitis, and P3 is even more specific for acute pancreatitis.
Low p-amylase is significant in exocrine pancreatic insufficiency. Also, a reduced p-amylase is suggestive of chronic pancreatitis. In pancreatic versus biliary disease, $P_3$ was found to be the best determinate. Isoamylases measured by wheat inhibition and cellulose acetate membrane electrophoresis revealed that increased p-amylase is indicative of acute pancreatitis, and $P_3$ isoamylase only occurs in acute pancreatitis. Positive $P_3$ by cellulose acetate electrophoresis has a positive efficiency of 93% and a negative efficiency of 100%. Thus, the use of a $P_3$ to exclude pancreatitis can alleviate expensive radiological procedures. Isoelectric focusing also showed an additional amylase fraction present in acute pancreatitis or pseudocysts similar to the $P_3$ fraction.

$P_3$ isoamylase has been evaluated in a group of 88 patients as an indicator of complications of acute pancreatitis. It was found that 92% of the patients who had complications (death, pseudocyst and recurrent pancreatitis) had an increase in $P_3$ levels; whereas, in the group of patients who had acute pancreatitis who had no complications at time of discharge only 7% had an increase in $P_3$.

During acute pancreatitis, it was found that the total and p-amylase levels were appreciably higher than the upper levels of control. Also s-amylase was either very low or not detected during the acute phase. During the recovery phase of the disease the enzyme values tended to return to normal.

Work has been done by several investigators on the inhibition of one of the amylase isoenzymes by a monoclonal antibody to allow the determination of the other isoenzyme. Steen developed an enzymatic colorimetric assay that utilizes two
monoclonal antibodies that inhibit s-amylase. It was found that bilirubin did interfere significantly with the assay.\textsuperscript{84} A monoclonal antibody coupled to plastic beads was developed by Svens. It extracted p-amylase from the sample. The catalytic activity of the s-amylase that was left was determined by its reaction with blocked p-nitrophenyl maltoheptaoside as a substrate.\textsuperscript{85} Another test being investigated utilizing monoclonal antibodies is based on the ability of two monoclonal antibodies to inhibit s-amylase nearly completely; therefore allowing the measurement of p-amylase by its catalytic action on the substrate, 4-nitrophenyl-alpha-D-maltoheptaoside.\textsuperscript{86} Another method employed a monoclonal antibody against s-amylase that was coupled indirectly to particles of polyvinylidene fluoride via a polyclonal goat anti-mouse immunoglobulin. This was used to remove s-amylase, and the resultant p-amylase was determined.\textsuperscript{58}

(3) Urinary

Urine amylase has come under scrutiny as to its value in the diagnosis of acute pancreatitis. It has been suggested by some to be abandoned. In evaluating urine levels [of amylase expressed as activity per volume (U/L) and activity per time excreted (U/h) and of amylase/creatinine clearance ratio] and serum levels (of amylase, elastase, lipase, and trypsinogen) at admission and repeatedly during hospitalization, individual serum assays have the highest sensitivity and specificity.\textsuperscript{87}

b. Other tests

(1) Lipase

The diagnosis of acute pancreatitis is most typically based on the clinical picture and elevated serum amylase. Total amylase determination has the advantage of
availability, technically simple, and at 24 hours of onset of pain as sensitive as lipase, pancreatic isoamylase, immunoreactive trypsin or elastase. However, after 24 hours it becomes one of the least sensitive. Lipase has the advantage of being more specific and almost as sensitive as amylase. Of interest the pancreas has four and a half times more lipase than amylase. The other tests (pancreatic isoamylase, immunoreactive trypsin and elastase) are not readily available to most hospital laboratories. These tests are best used in cases of difficult diagnosis. Other diagnostic procedures, such as ultrasonography, which aids in determining the extent of involvement of the biliary tract in the pancreatitis; and computed tomography (CECT), which is used in evaluating the extent of pancreatic necrosis, are helpful in determining the course of treatment. Early operation is suggested in severe acute pancreatitis.

In hyperamylasemic patients with the possibility of acute pancreatitis, serum lipase levels remain higher longer than either the pancreatic or total amylase. On hospital admission of acute pancreatitis patients, there is a poor correlation between lipase and serum amylase. Apple found that the efficiency of p-amylase, lipase, and total serum amylase was 94.1%, 76.5%, and 64.7%, respectively. Of p-amylase, lipase, total serum amylase, urine amylase, and serum amylase, Parodi reported that the best indicators for acute pancreatitis were p-amylase and lipase, with 96% and 95% accuracy. Pancreatic lipase appears to have the same temporal sequence as P3; however, at present, lipase is more convenient (more available and shorter turn around time).
The lipase levels are greater in alcoholic pancreatitis as compared to gall stone pancreatitis; whereas, in gallstone pancreatitis the amylase levels tend to be higher.\textsuperscript{91} It was found that the lipase values of proven pancreatitis ranged from 1011 to 25,706 U/L; whereas, in a group of 202 asymptomatic alcoholics the range was from 34 to 600 U/L.\textsuperscript{92} The ratio of lipase to amylase expressed in multiples of the upper normal limit of both indicated that a ratio of 2 or greater was indicative of alcoholic pancreatitis, and less than 2 implied a nonalcoholic etiology.\textsuperscript{91} Gumaste concluded that lipase was a better determinant for diagnosing acute alcoholic pancreatitis than amylase.\textsuperscript{92}

There have been reported some negative aspects of lipase testing. Tereault reported some surprising results of lipase values with the Ektachem 700 analyzer. Amylase has been reported to be more sensitive; while lipase has been reported to be more specific for pancreatitis. Out of 493 patients who had both tests ordered, it was found that 103 had values greater than the upper reference limit. Within this group lipase had 100% sensitivity and 84% specificity; while amylase had 72% sensitivity and 88% specificity.\textsuperscript{93} In rare cases, elevated serum lipase levels have been reportedly due to macrolipasemia.\textsuperscript{94}

(2) Pancreatic phospholipase A\textsubscript{2}

Pancreatic phospholipase A\textsubscript{2} (PLA\textsubscript{2}) has been evaluated as an indicator of acute pancreatitis since it is released from injured pancreatic acinar cells.\textsuperscript{95} PLA\textsubscript{2} was increased in 60\% of the chronic pancreatitis patients, 67\% of pancreatic cancer patients, and 27\% of non-pancreatic disease patients (most often in renal failure
patients). However, although present in acute pancreatitis, the pancreas is not the only source of PLA₂. PLA₂ levels showed no correlation with lipase or amylase but was related to the prognosis of acute pancreatitis. Elevated values have been seen with necrotizing pancreatitis and septicemia. Normal phospholipase A₂ values were seen in uncomplicated acute pancreatitis.

(3) Immunoreactive trypsin

Trypsin-α 1-proteinase inhibitor (alpha 1PI) complexes and trypsinogen comprise immunoreactive trypsin. Immunoreactive trypsin (IRT) was shown to minimally correlate with serum amylase in patients with acute pancreatitis; however, in patients who had persistent IRT with amylase values that had returned to normal, there were pancreatic complications, pseudocyst, and hemorrhagic pancreatitis. IRT may be useful as a prognostic tool. Lankisch summarized that the patient’s recovery pattern was best reflected by total amylase and p-amylase levels, and that trypsin in cases of delayed diagnosis was a good indicator. However, trypsin-α 1PI complexes can be elevated in conditions other than acute pancreatitis.

(4) Acute phase proteins

Certain acute phase proteins tests revealed that α-1-antitrypsin was the most sensitive index to diagnose chronic pancreatic disease, and C-reactive protein (CRP) reflected the stage of the disease best. These were independent of each other. However, all of the acute phase proteins (C-reactive protein, ceruloplasmin and alpha-1-antitrypsin) were all influenced by liver damage. Out of CT, CRP, PLA₂, and
amylase; CT and CRP were determined to be the best indicators of the prognosis of acute pancreatitis.\textsuperscript{102}

(5) Miscellaneous

Other diagnostic enzymes currently being investigated for their use in the diagnosis of pancreatitis are carboxylic ester hydrolase and elastase 1. Carboxylic ester hydrolase can be present in acute pancreatitis without an elevated amylase.\textsuperscript{103} It was also found that carboxylic ester hydrolase remained elevated in necrotizing pancreatitis for 10 days but decreased in acute interstitial pancreatitis. Amylase values in acute interstitial pancreatitis were higher than in those of necrotizing pancreatitis.\textsuperscript{104} Serum elastase 1 sensitivity was superior to lipase, p-amylase and immunoreactive trypsin when the cutoff was two times the upper limit; however, its specificity was less than lipase or p-amylase. In cases of renal insufficiency, it correlated with amylase and p-amylase.\textsuperscript{105} Serum elastase is reported to be increased to a greater extent than serum amylase in acute pancreatitis, and it remained elevated longer than amylase.\textsuperscript{106,107} Serum elastase-1, amylase, lipase, and trypsin-like immunoreactivity were all elevated at 24 hours in acute pancreatitis patients. However at 10 days only serum elastase-1 and trypsin remained elevated in 90\% of the acute pancreatitis patients.\textsuperscript{108} Ventrucci found amylase, pancreatic isoamylase, lipase, trypsinogen, and elastase 1 were all elevated on the first day of clinical symptoms. The greatest increase was seen in p-amylase; the lowest with elastase 1. With time, all serum enzyme levels decreased in a similar fashion except for the elastase 1, which remained elevated in all patients longer than the rest.\textsuperscript{109}
Serum enzyme values (amylase, pancreatic isoamylase, lipase, trypsinogen, and elastase 1) vary greatly in chronic pancreatitis patients during remission, and the majority of assays are normal (60%). In 67% of chronic pancreatitis elastase was within normal range. Likewise in pancreatic cancer highly variable enzyme levels are seen. Elastase 1 was the most frequently elevated enzyme (35%) in both groups. Gullo had previously found similar findings in chronic pancreatitis and carcinoma of the pancreas.

The most common symptom in acute pancreatitis is severe abdominal pain. In evaluating the severity of and determining the etiology, ultrasonography and computed tomography are useful and aid in deciding the treatment. ERCP is the most sensitive for diagnosing biliary lithiasis. Pancreatic necrosis has also been detected by C-reactive protein, PMN-elastase, and trypsinogen activation peptides.

c. Developmental tests

(1) Radioimmunoassays

Some of the first uses of monoclonal antibodies in diagnostic testing utilized radioactive isotopes. These methods were specific and sensitive; however, they have lost their importance in the clinical laboratory due to the storage and waste management of the radioactive materials. The structural differences between s-amylase and p-amylase are small but significant enough that they should enable a monoclonal antibody to be produced to each. A method by Fujita employing a monoclonal antibody to p-amylase has been developed utilizing a radioimmunoassay.
which shows negligible cross-reactivity to s-amylase. His test detects levels as low as 7 mg/L.\textsuperscript{113}

(2) Enzyme immunoassays

The problem of waste management and storage of radioactive isotopes has led the way for the development of a second generation of diagnostic tests that employ monoclonal antibodies. A variety of tests that employ monoclonal antibodies are now under investigation. Panozzo expressed apprehension in the use of a monoclonal antibody for diagnosing chronic pancreatic disease. He stated that the monoclonal under his investigation had limitations in its specificity although it was sensitive to pancreatic inflammation.\textsuperscript{114}

These tests belong to the group commonly called enzyme immunoassay (EIA). There are two main types of EIA: homogeneous, also called enzyme multiplied immunoassay technique (EMIT), and heterogeneous, also called enzyme linked immunosorbent assay (ELISA). In the EMIT, the antibody is added to the patient sample. The enzyme labeled antigen is then added. The sample competes with an enzyme labeled antigen for the antibody. If the antibody combines with the enzyme labeled antigen, the enzyme reaction is inhibited by the antibody either physically or by changing the conformation of the enzyme; thus the more antigen in the sample, the more enzyme labeled antigen that can react, which results in an increase in absorbance. The resultant color is directly proportional to the amount of antigen in the sample. Since there is no phase separation, the assays can be automated and quickly
performed. There is the disadvantage of interference from other constituents in the sample since there is no phase separation. This method is diagramed in Figure 1.

The ELISA requires phase separation of the antigen-antibody complex and the unbound constituents. There are two types of ELISA: the competitive, in which the resultant color is inversely related to the amount of antigen in the sample, and non-competitive, also called the sandwich assay, in which the resultant color is directly proportional to the amount of antigen in the sample. In the competitive assay the antibody is coated on the microwell. The sample with the antigen and an enzyme-conjugated antigen are added. The competition of the two antigens results in the amount of enzyme that is bound being inversely proportional to the amount of the antigen in the sample. The excess constituents are washed off, a substrate is added, and the resultant product is determined by a colored assay. This method is shown in Figure 2.

In the sandwich assay, there are two binding sites to the antigen. Again the well can be coated first with an antibody. The sample with the antigen is added, the unbound constituents washed off, and an enzyme-conjugated antibody (a second antibody) that is specific for a different epitope of the antigen is added. The excess is washed off, and again the substrate is added that results in a colored product being formed. The resultant color is directly proportional to the amount of the antigen present. Figure 3 demonstrates this reaction.

A number of researchers have produced pairs of monoclonal antibodies for either p-amylase or s-amylase. Diamandis determined p-amylase levels by the use of
Figure 1

Principle of the homogeneous enzyme immunoassay
Figure 2

Principle of the competitive heterogeneous enzyme immunoassay
Figure 3

Principle of the sandwich heterogeneous enzyme immunoassay
two monoclonal antibodies. One was immobilized to a microtiter well; the other was biotintylated. By the addition of streptavidin which was labeled with an europium chelator, the resulting fluorescence was measured, and the immunocomplex quantitated. The antibodies used showed low cross reactivity with s-amylase.\textsuperscript{115} Fujisawa also has reported success in using two monoclonal antibodies specific for p-amylase. This work also showed little cross reactivity to s-amylase.\textsuperscript{116} Another enzyme immunoassay that employed two monoclonal antibodies for detecting p-amylase, the latter being conjugated with peroxidase, was investigated by Fujisawa.\textsuperscript{116} Rosenblum has produced two monoclonal antibodies that bind p-amylase and do not block the active site of the enzyme. Good correlation with isoamylase determination by electrophoresis was obtained.\textsuperscript{117}

A test strip that is composed of monoclonal antibodies to s-amylase has been produced by Lorentz. It has been demonstrated to be useful in cases of slightly elevated amylase levels.\textsuperscript{118}

**B. Objectives**

The purpose of this research was to develop a solid phase enzyme immunoassay for determining p-amylase and s-amylase. This method involves the immobilization of cycloheptaamylose to the microwell. Subsequently, the p-amylase and s-amylase in the sample bind to the inhibitor. The respective levels of p-amylase and s-amylase are determined by the reaction of the specific monoclonal antibody against p-amylase or s-amylase. The prevalence of p-amylase and s-
amylase in hyperamylasemic patients were determined. The order of development and specific objectives were as follows:

1. Isolation and purification of p-amylase and s-amylase.

2. Production of monoclonal antibodies against the purified p-amylase and s-amylase by hybridoma technique.

3. Purification and characterization of the monoclonal antibodies.

4. Employment of the purified monoclonal antibodies for development of a solid phase enzyme immunoassay (EIA) for serum amylase.

5. Characterization of the developed solid phase EIA method and comparison with electrophoresis for determination of p-amylase and s-amylase in hyperamylasemic patients.
Chapter II

EXPERIMENTAL

A. MATERIALS:

All chemicals were Reagent Grade unless otherwise stated.

AAPER Alcohol and Chemical Co.: ethanol (USP)

Aldrich: 1,4 butanediol diglycidyl ether

Allied Chemical: dibasic sodium phosphate

American Type Culture Collection: NS1 (non-secreting mouse myeloma) cells

Becton Dickson: 1 cc and 3 cc syringes; 30 G, 25 G, 22 G, 18 G

Corning: 24 microwell plate

Dynatech Laboratories, Inc.: Immulon 1 Removawell strips

E.M. Science: ethyl ether anhydrous, sulfuric acid

Ethicon: ethicon silk 4.0

Falcon: 25 cm$^2$ 50 mL culture flask, 10 x 35 mm tissue culture dish; 15 mL sterile centrifuge tube

Fisher: sodium metaperiodate, sodium acetate, sodium hydroxide, dextrose, citric acid monohydrate, glycerin, boric acid

Gelman Instrument Company: Cellulose electrophoresis strips (Super Seprapore)
**Gibco:** Iscove's medium (IMDM), Fetal bovine serum (FBS);

penicillin/streptomycin (100x); Hybridoma-Serum Free Media

**JRH Biosciences:** HAT selective supplement (100x)

**J. T. Baker Chem Co.:** Iodine, sodium borohydride, cyclohexane

**Jackson Laboratories:** BALB/CByJ mice

**Kai:** Miltex Stainless steel sterile surgical blade

**Mallinckrodt:** sodium chloride, methanol, ammonium chloride

**Millipore:** 0.22 μm filter unit with filling bell

**Monoject, Division of Sherwood Medical:** heparinized capillary tubes

**Nunclon:** 96 microwell plate

**Oxford Labware:** critoseal

**Pharmacia Diagnostics:** Phadebas Amylase test tablet

**Sigma:** salivary α amylase, sodium phosphate (monobasic anhydrous),

Trizma Base (Tris[hydroxymethyl]aminomethane),

cycloheptaamylose (CHA, β cyclodextrin), Sepharose 6B,

phenylmethylsulfonyl fluoride, β glycerophosphate, amylose,

3,3’,5,5’-tetramethylbenzidine (TMB); Goat anti-Mouse IgG (whole molecule) Peroxidase Conjugate; Polyethylene glycol

(PEG);lipopolysaccharide (LPS); sodium bicarbonate; Ovalbumin

(OVA); polyoxyethylene-sorbitan monolaurate (Tween 20); HT media supplement: hypoxanthine 5 x 10^{-3} M thymidine 8 x 10^{-4} M,
50 X (HT); RPMI 1640 medium, with L-glutamine, without sodium bicarbonate; sodium azide, horse serum albumin, Protein A on Sepharose 4B fast flow, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), urea-hydrogen peroxide, barbital buffer, Sigma ImmunoType Mouse Monoclonal Antibody Isotyping Kit

Spectrum Medical Industries, Inc.: Spectra/Por dialyzing tubing 23 mm x 100 ft, m.w. cut off 6,000-8,000; and 10 mm x 50 ft, m.w. cut off 12,000-14,000

Voluntary Hospitals of America Supply Company: VHA Level I and VHA Level II (normal and abnormal human serum controls)

B. EQUIPMENT

Amicon Filtration System, model 12 and 52 with a YM10 filter
Beckman Appraise Densitometer
Beckman Microzone Cell Model R-101 Electrophoresis chamber
Beckman Model CDS-200 Computing Densitometer Scanner
Beckman Model TJ-6 centrifuge
Beckman sample applicator for cellulose acetate electrophoresis
Bio-Tek Instruments Model 207 Microplate reader
ISCO Model UA-5 Absorbance/Fluorescence Monitor
Nicolet 5PC FT-IR Spectrometer
C. Methods

1. Preparation of antigens

   a. Pancreatic amylase

      (1) Extraction

      The visible fat was trimmed from a normal human pancreas obtained from autopsy; the pancreas was then weighed. A buffer (containing 320 mg phenylmethylsulfonyl fluoride, 50 mM of β-glycerophosphate, and 150 mM of NaCl, pH 7.0) was added to the pancreas in a volume of 5:1 was placed in a Warring blender with the pancreas. The pancreas was homogenized with the buffer for 60 s. The resulting mixture was centrifuged at 4°C for 30 min at 5000 g. The fat rose to the top while the other debris remained at the bottom. The liquid portion was poured through four layers of gauze to retain the fatty material.

      (2) Purification

         (a) Production of cycloheptaamylose Sepharose-6B

         A sufficient quantity of Sepharose 6B was washed and dried on a course glass filter funnel with water to yield 20 grams. Twenty mL of 0.6 M NaOH containing 2 mg/mL sodium borohydride was added, followed by the addition of 20 mL of 1,4-butanediol diglycidyl ether. The mixture was divided evenly between two 50 mL plastic centrifuge tubes. These were mixed on a platform rocker for 8
h at 25°C. The mixture was poured onto a course glass filter funnel and washed with 4 L of deionized H₂O¹¹⁹. A schematic of the reaction is shown in Figure 4.

Twenty grams of the freshly prepared epoxy-activated Sepharose 6B that had been dried on a glass filter funnel was combined with 1500 mg of cycloheptaamylose and 60 mL of 0.1 M NaOH in two 250 mL Erlenmeyer flasks. The flasks were put on a shaker in a 45°C water bath for 19.5 hr. After incubation, the coupled Sepharose was washed for 30 min with approximately 4 L of deionized water on a glass filter funnel with slight aspiration. It was then washed with dextrose (25 mg/mL, approximately 500 mL) for 30 min with no aspiration. It was washed again with approximately 4 L of deionized water for 30 min with aspiration. It was finally washed with 4 L of 50 mM sodium acetate buffer pH 4.8 for 2 hr with no aspiration.¹²⁰,¹²¹ The final reaction is shown in Figure 5.

(b) Affinity chromatography

Equal portions (70 mL of each) of the above liquid portion (p-amylase homogenate) were mixed with a 50% solution of CHA-Sepharose in equilibration buffer (50 mM sodium phosphate and 50 mM of NaCl, pH 6.9) for 10 min in a 20 x 5 cm column. The column was then connected to a ISCO Model UA-5 Absorbance/ Fluorescence Monitor with the wavelength set at 280 nm. A sample of the eluent was tested to determine if any amylase was still present. One hundred µL of the sample were added to 100 µL of working substrate solution and
Figure 4

Activation of Sepharose with 1,4-butanediol diglycidyl ether
SEPHAROSE + 1,4-butenediol diglycidyl ether →

-CH₂-CH₂-O-(CH₂)₄-O-CH₂-CH₂-CH₂-CH₂

OH
Figure 5

Reaction of coupling CHA to sepharose
SEPAHAROSE-1,4-butandiol diglycidyl ether + CHA

SEPAHAROSE-1,4-butandiol diglycidyl ether-CHA
incubated at 37°C for 20 min. Then 850 µL of working iodine solution were added. Negative results for amylase appeared blue purple in color; whereas, positives appeared brown to dark yellow. The eluent was saved if it tested positive for amylase, and the extraction was repeated with reequilibrated CHA-Sepharose till no amylase was left in the eluent. The CHA-Sepharose-amylase was washed with the equilibration buffer till the base-line was stable. The amylase was eluted with 8.0 g/L of CHA in equilibration buffer. The eluent was monitored at 280 nm, and the eluent corresponding to the recorder peak was collected. The gel was reequilibrated by removing it from the column, placing it in a glass filter funnel, then washing it with the equilibration buffer (approx 100 mL). Next, it was washed with 1 N NaCl (approx 75 mL). Finally, it was washed and re-equilibrated with equilibration buffer (approximately 150 mL). The p-amylase was dialyzed in 6 L 10 mM PBS, pH 7.4, overnight with 2 changes of buffer to remove the CHA. All of the eluents were concentrated with an Amicon Ultrafiltration System, model 12 and 52 equipped with a YM10 filter. The concentrated p-amylase was stored in 1 mL aliquots at -80°C.

b. Salivary amylase

The s-amylase was purchased from Sigma. The solid (19 mg, 2500 units, 1450 units/mg protein) contained 1.724 mg protein and was reconstituted with 10 mL of 1 mM CaCl₂. The amylase was dialyzed with 6 L of 10 mM PBS, pH 7.4,
to remove the \((\text{NH}_4)_2\text{SO}_4\) in which it was stored. The buffer was changed 2 times every 8 hours. The s-amylase was frozen in 1 mL aliquots at -80°C.

2. Production of antibodies

   a. Immunization of mice

   A 5 week old BALB/CByJ male mouse was anesthetized by an intraperitoneal injection of pentobarbital (40-70 mg/Kg body weight). The animal was placed on its right side, and the area exposed on the left side of the abdomen was washed with 70% ethanol. A 0.5-1.0 cm incision was made aseptically into the left abdomen below the rib with small sterile scissors, exposing the spleen but not cutting through the peritoneum. Into the spleen, 100 µL of the p-amylase, which was diluted with 10 mM PBS, pH 7.4, to a concentration of 400 µg/mL (40 µg) were injected with a 1 mL syringe, 1/2 in 30 G needle. Using ethicon silk 4.0, two stitches were made aseptically to close the skin. The first booster of 20 µL of p-amylase, which was diluted with 10 mM PBS, pH 7.4, to a concentration of 2 mg/mL (40 µg) was given 11 days after initial injection using a 30 G needle via the tail vein. A second booster was given 13 days after the initial injection in the same manner as the first booster. The procedure was repeated with another mouse using s-amylase as the antigen.
b. **Titer determinations**

(1) **Antigen well preparation**

The Immulon 1 microtiter wells were coated with 200 μL of p-amylase at a concentration of 1 μg/mL, in a coating solution. After an incubation of a minimum of five hours (or overnight), the wells were washed 7 times with 10 mM PBS, pH 7.4, with 0.1% Tween followed by 7 times with deionized water. The wells were air-dried for approximately 10 min. Blocking of non-specific binding was performed with a 0.3% OVA in coating solution, 200 μL/well. After incubation for 2 hours, the wells were again washed 7 times with 10 mM PBS, pH 7.4, with 0.1% Tween, followed by 7 times with deionized water and air dried for approximately 10 min. The procedure was repeated using s-amylase as the antigen with another set of wells.

(2) **Titer assay**

A serum titer assay was performed to determine if sufficient antibodies were produced from the immunizations by the following procedure. A small incision was made in the tail of the mouse with a sterile stainless steel surgical blade, and a heparinized capillary tube was used to collect blood. The tube was filled approximately 2/3, then the end of the tube was sealed with critoseal and centrifuged at 1000 g for 10 min. Five μL of plasma was removed and serially diluted with 10 mM PBS, pH 7.4. Dilutions were 1:200; 1:400; 1:800; 1:1600; 1:3200; 1:6400; 1:12800; 1:25600; 1:51200; 1:102400; and 1:204800. To the
antigen-coated wells, 200 µL of each dilution were added and incubated at room temperature for at least 2 hours. Again they were washed 7 times with 10 mM PBS, pH 7.4, with 0.1% Tween, followed by 7 times with deionized water and air dried for approximately 10 min. Goat-anti-mouse IgG-HRPO, 200 µL/well, were added at a concentration of 5 µL diluted to 25 mL (1:5000) with 10 mM PBS, pH 7.4. The wells were incubated at room temperature for 1 hr. The wells were washed 7 times with 10 mM PBS, pH 7.4, with 0.1% Tween, followed by 7 times with deionized water and air dried for approximately 10 min. To the wells, 100 µL of buffered peroxide solution were added followed by 100 µL 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution. The wells were mixed on a rotator till a blue color appeared. The reaction was stopped with 50 µL of 2 N H₂SO₄. The wells were then read on a microplate reader at 450 nm, and the titer determined.

If the titer was not at least a hundredfold increase from the base line, the mouse was boosted in a similar manner every 2-3 weeks. A significant titer was expected to be at least a hundredfold difference of undiluted serum. If the titer was significant, the animal was sacrificed 1-3 days after the final boost. The spleen cells were harvested for cell fusion.

The same procedure was used utilizing s-amylase as the antigen in a second 5 week old BALB/CByJ male mouse.¹²³
c. Cell fusions

All dissection tools were sterilized in a hot oven (100°C) overnight. The day of the fusion, 70% ethanol was added to the dissection tray prior to use. The following were put in a 37°C water bath: MC-IMDM (25 mL), Serum Free Medium (40 mL), FBS (10 mL), LPS solution (0.2 mL of 4 mg LPS/mL of IMDM), HAT (50 X) (2.0 mL). A 50% warm PEG solution was prepared by autoclaving 1 g PEG for 15 min at 15 psi, then adding 1 mL RPMI 1640 when the PEG was warm.

NS-1 cells were grown in complete medium in a 37°C incubator with 100% humidity and 5% CO₂ in 50 mL Falcon tissue culture flask to yield 2 x 10⁷ - 5 x 10⁷ NS-1 cells (1/2 to 1/5 the number of spleen cells for fusion). On the day of fusion they were transferred to a 15 mL sterile centrifuge tube and centrifuged at 100 xg at room temperature for 5 min, and the supernatant was aspirated. They were resuspended in 5 mL Serum Free Media.

The mouse was boosted 1-3 days prior to fusion day. (If mouse was 8 weeks or less of age, the thymus was saved for thymocyte harvesting.) The mouse was sacrificed by cervical dislocation, and the abdomen was soaked with 70% ethanol. The spleen was carefully removed, using sterile technique. The spleen was placed in a 10 x 35 mm culture dish containing Serum Free Media and rinsed. It was transferred to another dish and rinsed again. The tissue was gently torn apart with forceps, minced with scissors, then mixed with the use of a 1-mL
plastic syringe. The spleen cell suspension was transferred through a sterile wire sieve, rubbing the tissue against the sieve, into a sterile 15 mL sterile centrifuge tube. A sample was taken and counted to determine the number of spleen cells. For fusion 1.0 x 10^8 spleen cells were needed. The suspension was centrifuged at 100 xg for 5 min at room temperature, and the supernate was removed by suction. To dissolve the erythrocytes that were present, 5 mL 0.85% NH₄Cl solution were added to tube and allowed to stand at room temperature for 5 min. The solution was centrifuged as before, and the supernatant aspirated. The pellet was resuspended in 5 mL Serum Free Media.

Thymocytes were obtained from a BALB/CByJ 5 weeks old mouse. (Thymocytes from the boosted mouse were used if under 8 weeks of age.) The mouse was sacrificed by cervical dislocation. The chest and throat area were sterilized by washing with 70% ethanol. The chest cavity was opened by standard procedure, and the thymus removed. It was rinsed with 3 mL Serum Free Media in a 10 x 35 mm tissue culture dish to remove as much blood as possible. It was transferred to a second dish and rinsed again. In a third culture dish, the gland was carefully torn with forceps to free the thymocytes into the 3 mL of the media. The tissue-cell mixture was further mixed with a 1 mL plastic syringe. The cell suspension was transferred through a sterile wire sieve, rubbing the thymus tissue against the sieve, into a sterile 15 mL tube, and a sample of the cell suspension was counted. Sufficient quantity was transferred to a 15 mL sterile tube to yield
2 X 10^8 thymocytes. The cell suspension was centrifuged at 100 xg for 5 min at room temperature. The supernatant was removed by suction. The cell pellet was resuspended in 1.4 mL 2X IMDM. The suspension was combined with 10 mL of FBS, 1 mL HAT (100X), and 0.2 mL of LPS solution (4 mg LPS/mL IMDM), plus 2.4 mL D.W. [Final volume 15 mL, Solution T]

Cell fusion was accomplished by the following schematic in Figure 6.

d. Hybridoma clone selection

A 96 well plate was prepared by adding 250 µL of complete medium that contained 1 x 10^4 to 10^5 thymocytes in each well. After approximately 10 days incubation after cell fusion, clones were selected that were smooth and homogeneous, had one center with well defined edges, and were not closer than 1.5 mm. The size of clone was between 0.5 mm and 1 mm. Using a dissecting scope in a closed plastic hood, each clone was siphoned with 50 µL pipette and dispersed one clone per well into the aforementioned 96 well plate. The clones were fed 1 drop (25 µL) of 1 x 10^4 to 10^5 thymocytes per well once a week, two times.124

e. Hybridoma clone propagation

When the media turned yellow, indicating good cell growth, the well was mixed and 150 µL were transferred into one well of a 24 well plate containing 1 mL of complete medium with 1 x 10^4 thymocytes. The 150 µL were replaced with
Figure 6

Cell fusion schematic
<table>
<thead>
<tr>
<th>NS-1 Cells</th>
<th>Spleen Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>(total 2 x 10^7)</td>
<td>(total 10^8)</td>
</tr>
<tr>
<td>in 5 mL Serum Free Media</td>
<td>in 5 mL Serum Free Media</td>
</tr>
</tbody>
</table>

| Ratio  | 1:5 |

Add spleen cells to NS1 in 50 mL tube

Centrifuge 500 rpm, 5 min; Remove supernate by suction

Take up 1 mL 50% PEG & add over 1 min

→ 0.1 mL added each 6 sec

Add 2 mL Serum Free Media over 2 min, stirring slowly

→ 0.1 mL added each 6 sec

Slowly add 7 mL more Serum Free Media the next 2-3 min

Centrifuge @ 1000 rpm, RT, 5 min

Remove the supernate by suction

Add 15 mL of Solution T to resuspend the pellet

Add to 25 mL 37°C MC-IMDM in a 50 mL tube; Mix for 3-4 min by slowly inverting

Dispense the 40 mL into culture dishes (1.5 mL per dish).

Incubate in 37°C incubator with 100% humidity and 5% CO₂.
new complete medium with thymocytes. When a well turned yellow the second time, a back up was made in another 96 well plate.

f. Hybridoma clone screening

Wells coated as previously described with either s-amylase or p-amylase were used to screen antibody production by the clones. After 10-14 days of growth, 100 μL of coating solution were pipetted into each well. Then 100 μL of supernatant of each clone well that had a yellow supernatant were added into each test well and incubated at room temperature for 2 hours. The wells were washed 7 times with 10 mM PBS, pH 7.4, with 0.1% Tween, followed by 7 times with deionized water, and dried for 10 min. Goat-anti-mouse IgG-HRPO, 200 μL/well, were added at a concentration of 5 μL diluted to 25 mL (1:5000) with 10 mM PBS, pH 7.4. The reaction was incubated at room temperature for 1 hr, then washed 7 times with 10 mM PBS, pH 7.4, with 0.1% Tween, followed by 7 times with deionized water, and finally air-dried for 10 min. To the wells, 100 μL of a buffered peroxide solution and 100 μL 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution were added. The plate was mixed on rotator till a blue color appeared. The reaction was stopped with the addition of 50 μL of 2 N H₂SO₄. The absorbance was read at 450 nm in a microplate reader; zero was set with a goat-anti-mouse IgG-HRPO blank.

Clones were selected that had good growth, were highly reactive with their respective antigen, and were negative for the opposite antigen. Also, a third group
was selected that showed good cross reactivity to both antigens. These were transferred to 24 well plates, which contained 2 mL/well of complete medium and incubated at 37°C in 100% humidity and 5% CO₂. From each of the above clones, hybridoma cells were frozen for back up.

g. Cultivation of antibodies

(1) Production of antibodies in vitro

As the hybridoma cells continued to grow, the supernatant of the culture medium turned acidic (yellow in color). At this time 1 mL of the supernatant was transferred aseptically to a 50 mL centrifuge tube and stored at -20°C till sufficient volume of supernatant was collected. One mL of complete medium was added to replace the supernatant that was removed. Two cell lines, 2PE10 and 2PC2, were also grown in Serum Free Hybridoma Media utilizing the same methodology.

(2) Production of antibodies in ascites fluid

An 8 week old BALB/CByJ male mouse was primed with 0.5 mL pristane intraperitoneal 3-4 days prior to hybridoma injection. Incomplete Freund’s Adjuvant was also injected intraperitoneal as a primer in some of the mice (0.25 mL, 7-10 days prior to hybridoma injection). The hybridoma cells that were grown in complete medium were counted and sufficient quantity was transferred to a centrifuge tube to yield 2 mL of 1 - 2 x 10⁷ cells/mL needed for injection. After centrifuging, the resultant supernatant was aspirated. Two mL of serum free medium was added to the pellet, and the cells were resuspended. The cells were
drawn into a 3 cc syringe with no needle attached. After the cells were in the syringe, a 25 G needle was attached, and the air was removed from the syringe and needle bringing the volume to 2 mL. The mouse's abdomen was washed with 70% alcohol, and 2.0 mL of 1 - 2 x 10^7 cells/mL were injected in intraperitoneal.

The mouse was checked for the production of ascites fluid. Beginning at one week and as late as three weeks depending on the growth rate of the cell line, the mouse would exhibit an extended abdomen. When the abdomen became distended, it was washed with 70% ethanol. The peritoneum was punctured with an 18 G needle on a 3 cc syringe, and the ascites fluid was collected drop-wise into a sterile test tube while applying gentle pressure to the sides of the abdomen of the mouse. The aspirate was frozen, and each succeeding tap of ascites fluid was frozen till the mouse expired. The procedure was repeated with each hybridoma cell line used.

h. Purification of antibodies

The cell culture supernatant or ascites fluid was centrifuged for 5-10 min at approximately 100 xg, and the supernatant measured. A Protein A-Sepharose column was washed with 10 mM PBS, pH 7.4, with the monitor set at 280 nm until the baseline was stabilized. The supernatant of the cell culture or ascites fluid was applied to the Protein A-Sepharose column. The first peak (1st fraction) containing the unwanted protein was discarded. The column was washed with 10 mM PBS, pH 7.4, until the baseline was again reached and stable. The column
was then washed with the elution buffer, and the effluent (2nd fraction) was collected into a flask. The elution was continued until the base line was again reached and was stable. The effluent in the flask was adjusted to pH 7.0 with 1.0 N NaOH. The effluent, which contained the IgG fraction, was transferred to dialyzing tubing then dialyzed twice in 6 L of 10 mM PBS, pH 7.4, for 8 hrs each. The dialysate was concentrated with an Amicon ultrafiltration system using a YM-10 filter, and the concentrate was stored in 1 mL aliquots at -20°C. The Protein-A Sepharose column was washed and stored in 0.02% sodium azide in 10 mM PBS, pH 7.4, at 4°C.\textsuperscript{126}

i. Conjugation of antibody with horseradish peroxidase (HRPO)

Sufficient HRPO was dissolved into 0.1 M sodium acetate buffer, pH 4.5, to give a final concentration of 10 mg/mL. Equal volumes of the HRPO solution and 0.1 M NaIO\textsubscript{4} were mixed and allowed to react at room temperature for 5 minutes. The mixture was then dialyzed in 6 L of 10 mM sodium acetate buffer, pH 4.0, overnight at 4°C with two changes of the solution. The antibody to be labeled with HRPO was put in a 0.1 M carbonate buffer, pH 8.3, to a final a concentration of 2 mg/mL. Equal volume of periodate oxidized HRPO and the antibody solution were mixed and allowed to stand at room temperature for 4 hours. The reaction was stopped with a NaBH\textsubscript{4} solution (1 mg/mL) in a volume ratio of 1:20 to the above reaction mixture. The antibody-HRPO was then dialyzed against 6 L of 10 mM PBS, pH 7.4, at 4°C for at least 6 hours. Horse serum
albumin was added to the sample to a final concentration of 10 mg/mL and
dialyzed once more in 6 L of 10 mM PBS, pH 7.4, at 4°C for at least 6 hours.
The antibody-HRPO was placed at 4°C for short term and -20°C for long term
storage.

j. Isotyping of antibodies

Each antibody was tested with Sigma ImmunoType Mouse Monoclonal
Antibody Isotyping Kit, stock No. ISO-1 according to the manufacturer’s
procedure. Two mL of antibody in a concentration of 1 μg/mL were used.

3. Assay conditions

a. Well coating studies

(1) CHA preparation studies

Into a 15 x 150 mm test tube, 1.6 g of CHA and 0.16 g of NaNO₂ were
placed in an ice water bath. To the CHA and NaNO₂ mixture, 5 mL of ice cold
concentrated nitric acid were added. The top of the test tube was sealed with
parafilm to trap the NO₂ that was produced. The mixture was maintained at 4°C
and stirred until all of the solid CHA was dissolved. One mL aliquots of the
mixture were taken at 8, 12, 24, 36, 42, and 48 hr. To the 1 mL aliquots, 8 mL
of ether were added to precipitate the CHA. The tube was centrifuged for 5 min at
100 xg, and the supernatant was removed. The precipitate was washed again with
4 mL of ether then centrifuged, and the supernatant removed. The precipitate was
allowed to air dry.127
Each of the oxidized CHA samples was analyzed by infrared analysis on a Nicolet 5PC FT-IR Spectrometer.

(2) CHA:BSA coupling studies

From the 36 hr oxidized CHA sample, a stock solution of 10 mg/mL deionized water was made. The CHA was activated by adding 0.1 M each of EDC and NHS in deionized water to $2.4 \times 10^{-3}$ M of CHA. This was mixed on a rocker at room temperature for 30 min. BSA diluted in 0.1 M carbonate buffer, pH 8.6, to a concentration of $1.5 \times 10^{-4}$ M was conjugated to the activated CHA and incubated on the rocker for 1 hr at 4°C in 1:1, 1:2, 1:4, and 1:8 ratios. This was repeated with increased EDC/NHS in the concentration of 0.1 M of each. This reaction is diagramed in Figure 7. The resulting CHA-BSA was dialyzed overnight in 6 L of 50 mM Tris buffer pH 8.5 with 2 changes of the buffer.

Tenfold dilutions were made of the CHA-BSA with the coating solution starting at 1 mg/mL. Two hundred μL aliquots were added to each well. The wells were incubated overnight at room temperature in a moist chamber.

b. Washing studies

Deionized water; 10 mM PBS, pH 7.4; and 10 mM PBS, pH 7.4, with 0.1% Tween (PBS-Tween) were chosen as possible washing solutions. Rinsing patterns performed were 3/3, 5/5, 7/7 of each of the washing solutions followed by deionized water. Rinses were performed after well coating, blocking, sample incubation and antibody incubation. The wells were completely filled with the
Figure 7

Reaction of coupling CHA to BSA
rinsing agent, then emptied, and after the final rinse were allowed to air dry in front of a fan for 10 min.128

c. Blocking studies

The wells were filled with 300 μL of blocking agent and incubated in a moist chamber. Blocking agents investigated were BSA, OVA, and skim milk in coating solution at the following concentrations: 0.3%, 0.5%, 1%, 3%, and 5%. Incubation was performed at room temperature and 37°C for 30, 45, 60, 90, and 120 min.128

d. Sample studies

(1) Sample diluent study

Samples were diluted with 10 mM PBS, pH 7.4, 10 mM PBS, pH 7.4, with 8 mmol/L CaCl₂, and 10 mM PBS, pH 7.4, with 0.1% skim milk. Each sample was serially diluted twofold with the diluent.128

(2) Sample size study

Two control sera (normal and abnormal) were serially diluted with 10 mM PBS, pH 7.4, with 0.1% skim milk (PBS-SM) and tested with the cross reacting 2PC12-HRPO antibody.

(3) Sample incubation study

Blanks (PBS-SM) and samples were incubated in a moist chamber at room temperature and 37°C for 30, 45, 60, 90, and 120 min.
**e. Antibody-HRPO incubation study**

The HRPO-conjugated antibody was diluted with 10 mM PBS, pH 7.4, and incubated in a moist chamber at room temperature and 37°C for 30, 45, 60, 90 and 120 min.

**f. Indicator system studies**

The substrate solution (solution A) was added proportionally to the well coating as established previously in this laboratory. The chromogen solution (solution B) was of equal volume to the substrate, and the 2 N H₂SO₄ was half volume to the previous two solutions. The color development was allowed to react for 5, 10, 15 and 20 min with serial dilutions of p-amylase and 2PC2-HRPO; likewise, with the s-amylase and 2S2F7-HRPO.

4. Studies of the performance characteristics of the enzyme immunoassay

a. Linearity studies

The standards (p-amylase and s-amylase) were added to human serum with a predetermined amylase level of less than 10 U/L. Each standard was made with the PBS-SM by twofold dilutions starting with a concentration of 1000 U/L of the respective isoamylase. These were tested with the appropriate HRPO-antibody.

b. Limit of detection studies

Twenty blanks were tested with 2PC2-HRPO and 2S2F7-HRPO.
c. Precision studies

Twenty determinations of commercially prepared normal pooled serum were determined with 2PC2-HRPO and 2S2F7-HRPO. Twenty assays using commercially prepared abnormal pooled serum were also performed with the two antibodies.

d. Cross reaction studies

The standards of p-amylase and s-amylase were each tested with the reciprocal antibody, p-amylase with 2S2F7-HRPO and s-amylase with 2PC2-HRPO.

5. Validation studies of human sera

a. Enzyme immunoassay

Twenty patient samples, normal control, and abnormal control were analyzed on Immulon 1 wells previously coated with 20 \( \mu \text{g/well} \) CHA:BSA that had been blocked with PBS-SM for 45 min at 37°C. The samples were diluted 1:5 with PBS-SM, and 200 \( \mu \text{L} \) were added to each well. These were incubated at room temperature for 60 min. They were then washed seven times with 10 mM PBS, pH 7.4, with 0.1% Tween followed by seven times with deionized water. The samples were incubated for 45 min at 37°C with 200 \( \mu \text{L} \) of each of the two antibodies, 2PC2-HRPO and 2S2F7-HRPO, that had been diluted to 1:600 and 1:700 respectively. The wells were again washed, and the two color developing solutions, A and B, were added. When a blue color developed, the color reaction
was stopped with the addition of 2 N H₂SO₄. The resulting yellow color was read on the microplate reader at 450 nm.

b. Total amylase

Total amylase determination of the patients samples were performed on a Beckman CX7 which measured the amount of NADH produced.

c. Electrophoresis

Gloves were worn to prevent any contamination. The number of applications of the sample onto the cellulose acetate strip was the equivalent of one application of a sample of an amylase of 500 U/L. This was made about 1.5 cm from the cathode end of the plate. Tris and barbital buffers were kept refrigerated then put into the anode and cathode chambers respectively. Electrophoresis was performed at 3 mA for 1.5 hours with the chamber in an ice bath. After electrophoresis, the strip was put into a closed chamber with one drop of water between the cellulose acetate and the glass plates to ensure that it lay perfectly flat. A Phadebas Amylase test tablet diluted with 3 mL of 8 mmol/L CaCl₂ was poured onto the electrophoresis plate and incubated at 40°C for 1.5 hours. After incubation, the plate was quickly washed under cold tap water, then put into methanol for 5 min to fix the blue bands to the cellulose acetate matrix, and then cleared in 30% cyclohexane in absolute ethanol for one min. The plate was dried in a warm oven for approximately 3 min. The strip was scanned at 595 nm, and the percentages of p-amylase and s-amylase were computed. 22,130
Chapter III

RESULTS

A. Preparation of antigens

Pancreatic amylase is unavailable commercially. A pancreas was homogenized and p-amylase was purified by affinity chromatography using CHA-Sepharose. The amylase was eluted with a solution of 0.8% CHA. This was later removed by dialysis. As seen in Table 1, not all of the amylase was totally extracted, still a large amount of p-amylase was obtained with a 58.8% yield from the original 100.65 grams of pancreatic tissue.

B. Production of antibodies

1. Immunization of mice

The immunization of the first p-amylase immunized mouse was particularly long due to the fact that NS1 cells were not available until the 17th week. Two mice were immunized with s-amylase. The hybridoma clone maintained from the first s-amylase mouse proved to produce too low of an antibody titer to be useful. The second s-amylase immunized mouse was maintained with boosters and was later utilized for cell fusion. However, due to a fungal infection, both p-amylase and s-amylase cell lines were lost from the fusions of the first p-amylase mouse and the second s-amylase mouse. Two more pairs of mice were immunized with p-amylase and s-amylase. The second p-amylase mouse gave rise to the cell line that was ultimately used for testing. The cell fusion from the third s-amylase mouse
Table 1. Summary of pancreatic amylase extraction and purification

<table>
<thead>
<tr>
<th>Sample</th>
<th>original volume/mL</th>
<th>final volume/mL</th>
<th>protein mg/mL</th>
<th>amylase Units/dL</th>
<th>total protein mg</th>
<th>total amylase U</th>
<th>specific activity Units/mg protein</th>
<th>yield %</th>
<th>purification folds</th>
</tr>
</thead>
<tbody>
<tr>
<td>homogenate</td>
<td>425</td>
<td>409</td>
<td>100</td>
<td>44100</td>
<td>40900</td>
<td>1803900</td>
<td>441</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1 CHA</td>
<td>21</td>
<td>5.5</td>
<td>338</td>
<td>930</td>
<td>1.9</td>
<td>5115</td>
<td>2692</td>
<td>0.03</td>
<td>6</td>
</tr>
<tr>
<td>#2 CHA</td>
<td>54</td>
<td>41</td>
<td>.653</td>
<td>85000</td>
<td>268</td>
<td>345000</td>
<td>130037</td>
<td>19.3</td>
<td>295</td>
</tr>
<tr>
<td>2nd extract #2</td>
<td>28</td>
<td>.653</td>
<td>86000</td>
<td>268</td>
<td>345000</td>
<td>130037</td>
<td>19.3</td>
<td>295</td>
<td></td>
</tr>
<tr>
<td>#3 CHA</td>
<td>144</td>
<td>50</td>
<td>.22</td>
<td>41500</td>
<td>11</td>
<td>207500</td>
<td>188636</td>
<td>11.5</td>
<td>428</td>
</tr>
<tr>
<td>2nd extract #3</td>
<td>13.5</td>
<td>.258</td>
<td>42000</td>
<td>3.5</td>
<td>567000</td>
<td>162931</td>
<td>3.1</td>
<td>369</td>
<td></td>
</tr>
<tr>
<td>#4 CHA</td>
<td>190</td>
<td>48</td>
<td>.266</td>
<td>43400</td>
<td>12.8</td>
<td>2083200</td>
<td>162750</td>
<td>11.5</td>
<td>369</td>
</tr>
</tbody>
</table>

NOTES:
no 3rd extract performed on #2 and #3
no 2nd extract performed on #4; had enough p-amylase; therefore, did not continue.
resulted in poor results due to the MC-IMDM having aggregates of media. The alternate mouse was boosted and used for cell fusion. The summary of the immunizations are shown in Table 2.

2. Titer determinations

All of the mice used exhibited a significant titer at 13 days. The continued immunization was due to other factors. The titers of P2 and P3 mouse are shown in Figure 8. The P2 mouse gave rise to the cell line used for testing (2PC2). The cells from the P3 mouse were never used. The titers of S3 and S4 mice are shown in Figure 9; mouse S4 gave rise to cell line 2S2F7 which was used in testing.

3. Cell fusions

The number of clones produced from the six cell fusions varied from 12 to 468. The ideal number to work with was approximately 100. Greater than this number of cells becomes unmanageable to maintain. Clone picking was usually performed only once. The one fusion that yielded 468 clones necessitated picking half of the clones one day and the remaining plates of clones the second day. Since clones that appeared later were the result of slower growing cell lines and were more likely to be contaminated from any cell(s) left from the initial clone picking, clones that grew later were not picked. Clones that were initially positive were screened later since hybridoma cells are unstable. Clones that were still viable and positive for the specific antibody decreased to approximately one fourth the original number. The summary of clone selections are shown in Table 2. Two cell lines that were positive for one amylase antigen and negative for the other were maintained (2PE10 and
Table 2. Summary of cell fusions

<table>
<thead>
<tr>
<th>Ag</th>
<th>boosters (days from spleen injection)</th>
<th>titer &amp; fusion (days from spleen injection)</th>
<th>clone picking (days from fusion)</th>
<th># of clones picked</th>
<th>transfer (days from clone picking)</th>
<th># of clones transferred</th>
<th>Ab screen (days from clone picking)</th>
<th>+ and growth</th>
<th>2nd Ab screen (days from clone picking)</th>
<th>+ and growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>12d, 13d, &amp; every 2-3 weeks thereafter</td>
<td>17 weeks</td>
<td>12, 13</td>
<td>468</td>
<td>14</td>
<td>336</td>
<td>24, 27, 36, 44</td>
<td>-</td>
<td>-</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>127 (↑1)</td>
</tr>
<tr>
<td>S1</td>
<td>11, 13</td>
<td>15</td>
<td>11</td>
<td>53</td>
<td>48</td>
<td>7</td>
<td>19</td>
<td>48, 6-P</td>
<td>10 (*2)</td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>12, 28, 42, 56, 84</td>
<td>86</td>
<td>10</td>
<td>6</td>
<td>84</td>
<td>24</td>
<td>20</td>
<td>6-P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>12, 13</td>
<td>15</td>
<td>10</td>
<td>77</td>
<td>3, 4, 5, 6</td>
<td>54</td>
<td>12</td>
<td>30, 10-S</td>
<td>22</td>
<td>15, 5-S</td>
</tr>
<tr>
<td>S3</td>
<td>12, 13,</td>
<td>15</td>
<td>10</td>
<td>12</td>
<td>(↑4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td>12, 13, 28</td>
<td>30</td>
<td>12</td>
<td>212</td>
<td>4, 6, 8</td>
<td>91</td>
<td>18, 28</td>
<td>35</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

*1 froze 24 best, lost in freezing technique; worked with 6 best-lost to fungus contaminate of media
*2 too low of antibody production
*3 lost to fungus contaminate of media (same time as P1 cells)
*4 fusion media had aggregates of hard media, too hard to pick clones
Figure 8


■: mouse 1; Y = 0.544 - 0.0812x log(X), r = 0.946
●: mouse 2; Y = 0.558 - 0.079x log(X), r = 0.984
Figure 9

Titer assay of anti-s-amylase by solid-phase enzyme immunoassay on microwell immobilization. Assay run in duplicate.
- ■: mouse 1; $Y=1.409-0.222x\log(X)$, $r=0.994$
- ●: mouse 2; $Y=1.116-0.169x\log(X)$, $r=0.962$
2PC2 for p-amylase; 2S2E4 and 2S2F7 for s-amylase). Two other cell lines (2PC12 and 2S1A7) that were cross reactive to both p- and s-amylase were also maintained to do the initial testing of the enzyme immunoassays.

4. Production of antibodies in vitro

As the hybridomas were grown to sufficient number for injection into the mouse to produce ascites fluid, the media that was removed and replaced with fresh media. The media that was removed was saved, stored at -20°C, and later purified to determine if a significant amount of antibody could be obtained by the in vitro cultivation of the cells. The amount varied from 1.4 μg/mL to 5.7 μg/mL of complete media. Two cell lines were also grown in Hybridoma Serum Free Media by Gibco and yielded 0 μg/mL and 5.8 μg/mL for 2PE10 and 2PC2, respectively. In vitro antibody production is summarized in Table 3.

5. Production of antibodies in ascites fluid

Ascites fluid production varied from 8 to 26 days from the intraperitoneal injection of the hybridoma cells with an average of 13 days. The total volume collected ranged from 3 mL to 17.2 mL. This figure reflects the amount collected not the total volume produced since some of the ascites fluid was lost during the tap. The average amount of fluid produced was 5.6 mL and 7.6 mL by priming with pristane and Incomplete Freund's Adjuvant, respectively. The average amount of antibody produced was 0.56 grams/mL of ascites fluid produced. The summary of all antibody production is in Table 3.
Table 3. Summary of antibody production

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Source</th>
<th>Time from I.P. cell injection to tap-volume days/mL</th>
<th>total liquid collected mL</th>
<th>antibody collected µg/mL</th>
<th>volume mL</th>
<th>grams</th>
<th>yield liquid collected g/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>2PE10</td>
<td>CM</td>
<td>130</td>
<td>368</td>
<td>@ 2</td>
<td>7.4x10³</td>
<td>5.7x10⁴</td>
<td></td>
</tr>
<tr>
<td>SF</td>
<td></td>
<td>85</td>
<td>0</td>
<td>@ 5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pr</td>
<td>14-3:16-3</td>
<td>6</td>
<td>886</td>
<td>@ 5</td>
<td>4.43</td>
<td>0.7383</td>
<td></td>
</tr>
<tr>
<td>IF</td>
<td>14-4</td>
<td>3</td>
<td>331</td>
<td>@ 5</td>
<td>1.32</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>2PC2</td>
<td>CM</td>
<td>120</td>
<td>94</td>
<td>6.8</td>
<td>6.4x10³</td>
<td>5.3x10⁴</td>
<td></td>
</tr>
<tr>
<td>SF</td>
<td></td>
<td>43</td>
<td>46</td>
<td>5.5</td>
<td>2.5x10³</td>
<td>5.8x10⁴</td>
<td></td>
</tr>
<tr>
<td>Pr</td>
<td>11-3.5:13-3.5</td>
<td>7</td>
<td>774</td>
<td>5</td>
<td>3.87</td>
<td>0.5529</td>
<td></td>
</tr>
<tr>
<td>IF</td>
<td>8-4.5:9-1.5</td>
<td>5</td>
<td>499</td>
<td>@ 6.5</td>
<td>3.2</td>
<td>0.644</td>
<td></td>
</tr>
<tr>
<td>IF</td>
<td>8-4.3:11-0.5:12-0.5</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2SIA7</td>
<td>CM</td>
<td>85</td>
<td>50</td>
<td>@ 5</td>
<td>2.5x10³</td>
<td>2.9x10⁴</td>
<td></td>
</tr>
<tr>
<td>Pr</td>
<td>9-4.5:11-2.8</td>
<td>6</td>
<td>688</td>
<td>@ 5</td>
<td>3.44</td>
<td>0.5733</td>
<td></td>
</tr>
<tr>
<td>2PC12</td>
<td>CM</td>
<td>50</td>
<td>56</td>
<td>@ 5</td>
<td>2.8x10³</td>
<td>5.6x10⁴</td>
<td></td>
</tr>
<tr>
<td>Pr</td>
<td>9-4.5:11-4</td>
<td>7</td>
<td>651</td>
<td>@ 5</td>
<td>3.26</td>
<td>0.4657</td>
<td></td>
</tr>
<tr>
<td>IF</td>
<td>12-4:14-4.3:16-4.5:18-4.4</td>
<td>17.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2S2F7</td>
<td>CM</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pr</td>
<td>17-2.6:19-3.3</td>
<td>4</td>
<td>399</td>
<td>@ 5</td>
<td>2.0</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>IF</td>
<td>18-1:21-2.5:24-1:26-3.3:27-1</td>
<td>7.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2S2E4</td>
<td>CM</td>
<td>65</td>
<td>18</td>
<td>@ 5</td>
<td>9.0x10³</td>
<td>1.4x10⁴</td>
<td></td>
</tr>
<tr>
<td>Pr</td>
<td>26-1.3:28-2.2</td>
<td>3.5</td>
<td>383</td>
<td>@ 5</td>
<td>1.92</td>
<td>0.549</td>
<td></td>
</tr>
</tbody>
</table>

abbreviations: (CM) complete media, (SF) serum free media, (Pr) ascites fluid from pristane primer, (IF) ascites fluid from Incomplete Freund's Adjuvant primer, (@) approximately

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6. **Purification of antibodies**

The media in which the hybridoma cells were grown and the ascites fluid were both purified by affinity chromatography with Protein A-Sepharose using a citrate buffer of pH 3.0. The elutions of 2PC2 and 2S2F7 are seen in Figures 10 and 11, respectively.

7. **Isotyping of antibodies**

All three antibodies (2PC2, 2S2F7 and 2PC12) were determined to be IgG2b by the Sigma ImmunoType Mouse Monoclonal Antibody Isotyping Kit.

C. **Assay conditions**

1. **Well coating studies**

Due to cycloheptaamylose’s circular structure of seven glucose units, as seen in Figure 12, it acts as an inhibitor of amylase. In order to coat the microwells with cycloheptaamylose it was necessary to attach it to a protein. This was accomplished by first oxidizing at least one of the hydroxyl groups to a carboxyl group and then connecting it to the protein by an amide linkage. The circular structure of cycloheptaamylose also had to be maintained to insure that it remained an inhibitor of amylase and did not instead become a substrate for amylase. The results of the oxidation of cycloheptaamylose at 8, 12, 24, 36, 42, 48 and 84 hr are shown by the graphs of the infrared analysis in Figures 13 and 14. The appearance of an additional peak at 1750 wave numbers starts to appear at 8 hr. It becomes equal in length to the peak at 1650 at 36 hours. This concurs with the formation of a carboxyl group, with the peak at 1650 due to the presence of the carbonyl group in a semiacetyl formation.
Figure 10

The elution profile of anti-p-amylase from protein A sepharose chromatography
Figure 11

The elution profile of anti-s-amylase from protein A sepharose chromatograph
Figure 12

Structure of cycloheptaamylose
Figure 13

Infrared analysis of the oxidation of cycloheptaamylose
A. 0 hours, B. 8 hours, C. 12 hours, and D. 24 hours
Figure 14

Infrared analysis of the oxidation of cycloheptaamylose
A. 36 hours, B. 42 hours, C. 48 hours, and D. 84 hours
The peak at 1150 concurs with the ether linkage that results in the ring structure of cycloheptaamylose. When the oxidation was allowed to go for 84 hr, the peak at 1650 began to deteriorate. Oxidation time was chosen to be 36 hr since the two peaks due to carbonyl and carboxyl groups were equal in length, and it was only necessary to oxidize one hydroxyl group on cycloheptaamylose.

Of the ratios 1:1, 1:2, 1:4, and 1:8 of BSA to CHA, the highest reacting ratio was 1:1, as seen in Figure 15. Since the absorbance of all of the ratios was low, a mean of 0.156, further studies were performed to rectify this.

Activation of the carboxyl group was undertaken with carbodiimide by the formation of an O-acylurea. However, in an aqueous media this intermediary is subject to hydrolysis. The presence of N-hydroxy-succinimide (NHS) results in the formation of an active ester that is more hydrolysis resistant.\textsuperscript{128,129} By increasing the concentration of EDC and NHS from 1 mM to 0.1 M, the readings were increased from a mean of 0.156 to 0.565. The best ratio of BSA to CHA was chosen to be 1:1 at a concentration of 20 µg/well since this was the only ratio with a positive slope. The results are shown in Figure 16.

2. Washing studies

Three washing agents were tested: deionized water, 10 mM PBS, pH 7.4, with and without 0.1% Tween. Each of these was followed by an equal number of washings with deionized water. Figure 17 shows that, washing 7 times PBS-Tween followed by 7 times with deionized water gave the greatest difference between the sample with the antigen and the blank.
Figure 15

Optimization of coupling oxidized cycloheptaamylose to bovine serum albumin with 1 mM aqueous 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide for assay in solid-phase enzyme immunoassay on microwell immobilization. Assay run twice.

■: 1BSA:1CHA; Y = 0.176 + 6.7x10^4(X), r = 0.288
●: 1BSA:2CHA; Y = 0.156 + 2.0x10^4(X), r = 0.451
♦: 1BSA:4CHA; Y = 0.143 + 2.0x10^4(X), r = 0.534
▲: 1BSA:8CHA; Y = 0.136 - 9.2x10^3(X), r = 0.253
Figure 16

Optimization of coupling oxidized cycloheptaamylose to bovine serum albumin with 0.1 M aqueous 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide for assay in solid-phase enzyme immunoassay on microwell immobilization. Assay run twice.

■: 1BSA:1CHA; Y=0.571+2.0x10^4(X), r=0.266
●: 1BSA:2CHA; Y=0.568-2.0x10^4(X), r=0.604
♦: 1BSA:4CHA; Y=0.625-6.0x10^4(X), r=0.793
▲: 1BSA:8CHA; Y=0.581-4.9x10^4(X), r=0.044

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Figure 17

Washing study on solid-phase enzyme immunoassay on microwell immobilization.
Assay run twice.

Washing agent on samples:
- ■: deionized water; \( Y=0.307-1.6 \times 10^{-2}(X), r=0.459 \)
- ●: 10 mM PBS, pH 7.4; \( Y=0.267-2.0 \times 10^{-3}(X), r=0.449 \)
- ♦: 10 mM PBS, pH 7.4, with 0.1% Tween;
  \( Y=-0.100+8.4 \times 10^{-2}(X), r=0.999 \)

Washing agent on blanks:
- □: deionized water; \( Y=0.103+4.2 \times 10^{-2}(X), r=0.469 \)
- ○: 10 mM PBS, pH 7.4; \( Y=0.262-1.8 \times 10^{-2}(X), r=0.554 \)
- ♦: 10 mM PBS, pH 7.4, with 0.1% Tween; \( Y=0.138+0.0(X), r=0.999 \)

Net difference of sample minus blank:
- ⚫: deionized water; \( Y=-0.112+3.3 \times 10^{-2}(X), r=0.95 \)
- ⭕: 10 mM PBS, pH 7.4; \( Y=-0.003+1.6 \times 10^{-2}(X), r=0.686 \)
- ⭧: 10 mM PBS, pH 7.4, with 0.1% Tween;
  \( Y=-0.111+5.0 \times 10^{2}(X), r=0.999 \)
3. **Blocking studies**

The three blocking agents tested were BSA, OVA, and skim milk in coating solution at the following concentrations: 0.3%, 0.5%, 1%, 3%, and 5%. The results are shown in Figure 18. BSA and skim milk gave the greatest difference between the sample with the antigen and the blank with BSA being slightly better. OVA appeared to inhibit the activity of the antigen since little difference was observed between the sample and the blank. Since both 0.5% and 3% gave the best net difference between the sample and the blank, they were further tested at room temperature and 37°C. The results are in Figures 19-22. Of these testings, 0.5% BSA in coating solution for 45 min at 37°C was chosen for blocking the unbound spaces on the polystyrene surface.

4. **Sample studies**

   a. **Sample diluent study**

   The diluents investigated consisted of 10 mM PBS, pH 7.4; PBS-SM; and 10 mM PBS, pH 7.4, with 8 mmol/L CaCl₂. Although chloride ions are necessary for amylase activity, and calcium ions are necessary for the structural integrity of the enzyme, protein appears to also be necessary to maintain the binding conditions of amylase to the inhibitor CHA. Figure 23 shows that PBS-SM was the best diluent.

   b. **Sample size study**

   The abnormal control sera used to test the diluent, as seen in Figure 23, showed lower activity undiluted than when diluted 1:2 or 1:4. The 1:4 dilution in fact
Study of dilutions of blocking agents on solid-phase enzyme immunoassay on microwell immobilization. Assay run at room temperature twice.

Blocking agent with samples:
- ■: 10 mM PBS, pH 7.4, with BSA;
- ●: 10 mM PBS, pH 7.4, with 0.1% skim milk;
- ♦: 10 mM PBS, pH 7.4, with OVA

Blocking agent with blanks:
- □: 10 mM PBS, pH 7.4, with BSA;
- ○: 10 mM PBS, pH 7.4, with 0.1% skim milk;
- ◊: 10 mM PBS, pH 7.4, with OVA

Net difference of sample minus blank:
- ///: 10 mM PBS, pH 7.4, with BSA;
- ///: 10 mM PBS, pH 7.4, with 0.1% skim milk
- ///: 10 mM PBS, pH 7.4, with OVA
Figure 19

Blocking incubation study of 0.5% bovine serum albumin at room temperature on solid-phase enzyme immunoassay on microwell immobilization.
Assay run twice.
■: sample; □: blank; ★★★: sample - blank

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Figure 20

Blocking incubation study of 0.5% bovine serum albumin at 37°C on solid-phase enzyme immunoassay on microwell immobilization. Assay run twice.

■: sample; □: blank; ✖️: sample - blank

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Figure 21

Blocking incubation study of 3% bovine serum albumin at room temperature on solid-phase enzyme immunoassay on microwell immobilization. Assay run twice. ■: sample; □: blank; ⨃: sample - blank

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Figure 22

Blocking incubation study of 3% bovine serum albumin at 37°C on solid-phase enzyme immunoassay on microwell immobilization. Assay run twice.
■: sample; □: blank; ★★★: sample - blank
Sample diluent study on solid-phase enzyme immunoassay on microwell.
Assay run twice.
Diluents:
- ■: 10 mM PBS, pH 7.4;
  \[ Y = 0.587 + 2.3^4(X), \ r = 0.074 \]
- ●: 10 mM PBS, pH 7.4 with 8 mM CaCl₂;
  \[ Y = 0.572 - 2.1x10^4(X), \ r = 0.371 \]
- ♦: 10 mM PBS, pH 7.4 with 0.1% skim milk;
  on data points 1-85: \[ Y = 0.246 + 4.3x10^3(X), \ r = 0.926 \]

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showing the highest absorbance. The fact that the activity increases with a smaller sample size was confirmed by testing twofold dilutions of the normal control sera along with the abnormal control sera, seen in Figure 24. From these results the sample size was reduced to 40 μL instead of 200 μL/well. A 1:5 dilution of each sample was made with the PBS-SM (40 μL sample plus 160 μL PBS-SM) when performing the assay.

c. Sample incubation study

Samples of normal control serum and blanks of the diluent were incubated at room temperature for 30, 45, 60, 90, and 120 min. These results, plus the net difference between the sample and blank, are shown in Figure 25. This study was also performed at 37°C, and the results are illustrated in Figure 26. Room temperature provided the best readings with a 60 min incubation determined as the best incubation time.

5. Antibody-HRPO studies

The HRPO conjugated antibodies (2PC12, 2PC2, and 2S2F7) were serially diluted with 10 mM PBS, pH 7.4, and incubated in a moist chamber at room temperature and 37°C for 30, 45, 60, 90 and 120 min. The results of the serial dilutions of the HRPO-antibodies are seen in Figures 27-29. From these 2PC12 was diluted 1:750 and used for testing the assay procedure. A 1:600 dilution was made for 2PC2, and a 1:700 dilution was made for 2S2F7. The results of incubation times of 30, 45, 60, 90 and 120 min at room temperature are shown in Figure 30. Figure 31 shows the results of the 37°C incubation temperature at the same incubation times.
Figure 24

Sample size study on solid-phase enzyme immunoassay on microwell.
Assay run twice.
■: normal control; Y = 0.797 - 2.1 \times 10^{-3}(X), r = 0.901
●: abnormal control; on points 21-85: Y = 0.655 + 3.0 \times 10^{-3}(X), r = 0.992
Figure 25

Sample incubation study at room temperature on solid-phase enzyme immunoassay on microwell immobilization. Assay run twice.
■: sample; □: blank; ☒: sample - blank
Figure 26

Sample incubation study at 37°C on solid-phase enzyme immunoassay on microwell immobilization. Assay run twice.
■: sample; □: blank; ###: sample - blank

■: sample; $Y = 0.852 + 1.0 \times 10^{-4}(X), r = 0.847$

□: blank; $Y = 0.570 - 8.7 \times 10^{-5}(X), r = 0.845$

★★: sample - blank;
Figure 28

Antibody dilution study of anti-p-amylase-HRPO (2PC2) on solid-phase enzyme immunoassay on microwell immobilization. Assay run in duplicate.

■: sample; \( Y = 1.136 - 8.9 \times 10^4(X) \), \( r = 0.940 \)

□: blank; \( Y = 0.836 - 7.6 \times 10^4(X) \), \( r = 0.860 \)

△△: sample - blank
Figure 29

Antibody dilution study of anti-s-amylase-HRPO (2S2F7) on solid-phase enzyme immunoassay on microwell immobilization. Assay run in duplicate.

■: sample; Y=1.127-5.5x10^4(X), r=0.827
☐: blank; Y=0.470-1.5x10^4(X), r=0.450
★★: sample - blank
Figure 30

Antibody incubation study at room temperature on solid-phase enzyme immunoassay on microwell immobilization. Assay run twice.

■: sample; □: blank; ☒: sample - blank

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Figure 31

Antibody incubation study of antibody at 37°C on solid-phase enzyme immunoassay on microwell immobilization. Assay run twice.
■: sample; □: blank; ■■: sample - blank
The antibody appeared to bind better at 37°C than room temperature; therefore, a 45 min incubation at 37°C was selected as the best incubation for the antibody-HRPO.

6. Indicator system studies

The results of solution A and B incubation at 5, 10, 15 and 20 min with serial dilutions of p-amylase and 2PC2-HRPO are shown in Figure 32. The results of incubation with s-amylase and 2S2F7-HRPO are shown in Figure 33. Color development was best between 15 and 20 min.

D. Study of the performance characteristics of the enzyme immunoassay

1. Linearity studies

The assay procedure that was employed in testing for p-amylase and s-amylase is summarized in Table 4. The results of the serial dilution of p-amylase and 2PC2-HRPO are seen in Figure 34. Pancreatic amylase determinations were linear from 8 U/L up to the 1000 U/L tested. Salivary amylase determinations were linear from 16 U/L to the 1000 U/L tested. The linearity of 2S2F7 are shown in Figure 35.

2. Limit of detection studies

Twenty blanks were run simultaneously with standards for p-amylase and s-amylase. The standard deviation calculated for p-amylase was 0.064 and for s-amylase was 0.047. Following the recommendation of the International Federation of Clinical Chemistry (IFCC), the limits of detection equal 2.6 x S.D., which are 0.166 and 0.122 for p-amylase and s-amylase, respectively. From the standard curve which was run in the same assay with the blanks, the limits of detection were determined to be 8 U/L for p-amylase and 16 U/L for s-amylase.
Figure 32

Color development time study of p-amylase solid-phase enzyme immunoassay on microwell immobilization. Assay run twice.

Incubation times:

- ■: 5 min; \( Y = 0.216 + 8.4 \times 10^4(X) \), \( r = 0.941 \)
- ●: 10 min; \( Y = 0.428 + 1.6 \times 10^3(X) \), \( r = 0.912 \)
- ♦: 15 min; \( Y = 0.535 + 2.1 \times 10^3(X) \), \( r = 0.980 \)
- ▲: 20 min; \( Y = 0.603 + 2.7 \times 10^4(X) \), \( r = 0.987 \)

slope vs time: \( Y = 3.5 \times 10^4 + 3.3 \times 10^4(X) \), \( r = 0.932 \)
Concentration of Amylase (U/L) vs. Absorbance (450 nm)
Figure 33

Color development time study of s-amylase solid-phase enzyme immunoassay on microwell immobilization. Assay run twice.

Incubation times:

- ■: 5 min; $Y = 0.277 + 1.1 \times 10^{-3} (X)$, $r = 0.946$
- ●: 10 min; $Y = 0.341 + 3.2 \times 10^{-3} (X)$, $r = 0.979$
- ♦: 15 min; $Y = 0.383 + 3.3 \times 10^{-3} (X)$, $r = 0.947$
- ▲: 20 min; $Y = 0.583 + 4.9 \times 10^{-3} (X)$, $r = 0.910$

slope vs time: $Y = 2.5 \times 10^{-4} + 2.3 \times 10^{-4} (X)$, $r = 0.953$
Table 4. Summary of the assay conditions for enzyme immunoassay for p-amylase and s-amylase determination

<table>
<thead>
<tr>
<th>Substance</th>
<th>Time</th>
<th>Temperature</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
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<tr>
<td>washing</td>
<td>7</td>
<td></td>
<td>10 mM pH 7.4 -0.1%</td>
<td>400 μL</td>
</tr>
<tr>
<td>followed by:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>washing</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>well coating of CHA</td>
<td>5-24</td>
<td>room temperature</td>
<td>20 μg/well</td>
<td>200 μL</td>
</tr>
<tr>
<td>blocking</td>
<td>45</td>
<td>37°C</td>
<td>0.5%</td>
<td>300 μL</td>
</tr>
<tr>
<td>antigen</td>
<td>60</td>
<td>room temperature</td>
<td></td>
<td>40 μL</td>
</tr>
<tr>
<td>antigen diluent</td>
<td></td>
<td></td>
<td>0.1% in 10 mM pH 7.4</td>
<td>160 μL</td>
</tr>
<tr>
<td>antibody-HRPO</td>
<td>45</td>
<td>37°C</td>
<td>1/600 2PC2-HRPO 1/700 2S2F7-HRPO</td>
<td>200 μL</td>
</tr>
<tr>
<td>antibody diluent</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>color development</td>
<td>15-20</td>
<td>room temperature</td>
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<td>100 μL</td>
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<td></td>
</tr>
<tr>
<td>solution B</td>
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<tr>
<td>H₂SO₄</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

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Figure 34

Linearity of anti-p-amylase-HRPO (2PC2) on solid-phase enzyme immunoassay on microwell immobilization. Assay run three times.

\[ Y = 0.181 + 0.366 \log(X), \quad r = 0.972 \]
Figure 35

Linearity of anti-s-amylase-HRPO (2S2F7) on solid-phase enzyme immunoassay on microwell immobilization. Assay run three times.

\[ Y = -0.092 + 0.570 \log(X), \quad r = 0.933 \]
3. **Precision studies**

Twenty replicates of commercially prepared pooled normal serum and abnormal serum were assayed in the same experiment for p-amylase and s-amylase. A summary of the statistical analysis of the assays are in Table 5 for p-amylase and Table 6 for s-amylase. The coefficient of variation (C.V.%) for p-amylase normal serum was 7.79%, and 6.28% for abnormal sera. For s-amylase the C.V.% was 9.42% and 9.29% for normal and abnormal sera, respectively.

4. **Cross reactivity studies**

Initial screening of 2PC2 showed an absorbance reading of 0.454 against a blank of complete media with p-amylase precoated on the wells at a concentration of 1 µg/mL and 0.006 against a blank of complete media with s-amylase precoated on the wells at a concentration of 1 µg/mL. Original screening of 2S2F7 showed an absorbance reading of 0.404 against a blank of complete media with s-amylase precoated on the wells at a concentration of 1 µg/mL and 0.032 against a blank of complete media with p-amylase precoated on the wells at a concentration of 1 µg/mL. Later assays using s-amylase and 2PC2 and p-amylase and 2S2F7 resulted in determinations below the level of detection.

E. **Validation studies**

Thirty-two serum patient samples along with a normal control, and abnormal control were analyzed on Immulon 1 wells previously coated with 20 µg/well CHA:BSA that had been blocked with 0.5% BSA in coating solution for 45 min at 37°C. The samples were diluted 1:5 with PBS-SM, and 200 µL was added to each
Table 5. Precision studies of p-amylase determination

<table>
<thead>
<tr>
<th></th>
<th>normal</th>
<th>abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>sample</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>n</td>
<td>1.253</td>
<td>1.331</td>
</tr>
<tr>
<td>mean</td>
<td>1.469</td>
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<td>max</td>
<td>1.059</td>
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<tr>
<td>S.D.</td>
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<td>.084</td>
</tr>
<tr>
<td>C.V.%</td>
<td>7.79</td>
<td>6.28</td>
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</table>
Table 6. Precision studies of s-amylase determination

<table>
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<th>sample</th>
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</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>mean</td>
<td>0.866</td>
<td>0.580</td>
</tr>
<tr>
<td>max</td>
<td>1.009</td>
<td>0.683</td>
</tr>
<tr>
<td>min</td>
<td>0.741</td>
<td>0.489</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.082</td>
<td>0.054</td>
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<td>C.V. %</td>
<td>9.42</td>
<td>9.29</td>
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</table>
well. These were incubated at room temperature for 60 min. They were then washed seven times with PBS-Tween followed by seven times with deionized water. The samples were incubated for 45 min at 37°C with 200 μL of each of the two antibodies, 2PC2-HRPO and 2S2F7-HRPO, that had been diluted to 1:600 and 1:700 respectively. The wells were again washed, and the two color developing solutions, A and B, were added. When a blue color developed, the color reaction was stopped with the addition of 2 N H₂SO₄. The resulting yellow color was read on the microplate reader at 450 nm. The summary of admitting diagnosis, total amylase, cellulose acetate electrophoresis and EIA for p-amylase and s-amylase are shown in Table 7. Linear regression of the results gave an intercept of 82.060, a slope of 0.460 and correlation coefficient of 0.820 for p-amylase; however, samples number 6, 77, and 201 included P3 amylase which is not detected by the EIA. Using just the values of P1 and P2 for the p-amylase value for electrophoresis, the intercept was 21.310, the slope was 1.152, and the correlation coefficient was 0.920. For s-amylase the intercept was -1.25, slope 1.15, and correlation coefficient was 0.867.
Table 7. Comparison of electrophoresis and enzyme immunoassay of p-amylase and s-amylase on human serum samples

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<tr>
<th>Sample #</th>
<th>admitting diagnosis</th>
<th>total amylase</th>
<th>electrophoresis p-amylase</th>
<th>EIA p-amylase</th>
<th>electrophoresis s-amylase</th>
<th>EIA s-amylase</th>
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<td>2</td>
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<td>153</td>
<td>15</td>
<td>361</td>
<td>138</td>
<td>175</td>
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<tr>
<td>3</td>
<td>beaten head &amp; face</td>
<td>524</td>
<td>&lt;10</td>
<td>31</td>
<td>524</td>
<td>656</td>
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<td>6</td>
<td>chest pains</td>
<td>4000</td>
<td>3000 (total P)</td>
<td>1299</td>
<td>1000</td>
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<tr>
<td></td>
<td></td>
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<td>1200 (P, &amp;P,)</td>
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<td>total amylase</td>
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<td>EIA p-amylose</td>
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Table 7 Comparison...serum samples (continued)

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<th>Sample #</th>
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</table>

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Chapter IV

DISCUSSION

A. Amylase

1. Purification

Normal pancreatic tissue obtained from autopsy was purified by various other procedures that included (NH₄)₂SO₄ precipitation, acetone and sodium acetate extraction; however, the procedure outlined in the methodology gave the best yield. The main essential was that all of the extractions had to be performed as quickly as possible while maintaining the enzyme’s stability in a concentrated solution of pH 7.0 to 8.0, and stored at 4°C or less.

2. Amylase standards

The purified p-amylase was stored at -80°C with some smaller aliquots at -20°C and 4°C. Activity did decrease over the period of a year. The -80°C preserved the amylase best while 4°C preserved it the least. Dilutions of the enzyme were later added to human serum with an amylase value of 6 U/L for the establishment of the standard curves. Storage of the enzyme appears to be better in a higher protein media than the 10 mM PBS, pH 7.4, buffer. The p-amylase and s-amylase standards were assayed first in the PBS-SM diluent; however, their activity was not as great as when they were added to either serum free amylase, serum that had been extracted with CHA-Sepharose, or serum with an amylase level of less than 10 U/L. The serum with the amylase level of less than 10 U/L gave the best reactivity for the isoamylases. Electrophoresis of the p-amylase and s-amylase in 10 mM PBS, pH 7.4, were
performed. The p-amylase sample gave one distinct sharp band. The s-amylase had a few much smaller peaks on the scan of the cellulose acetate electrophoresis. The s-amylase was purchased commercially and not subjected to Protein A Sepharose purification. Of the two assays, s-amylase determinations had a higher detection limit, a lower correlation coefficient, and the C.V. % for the normal and abnormal control sera were higher than for p-amylase.

3. Chemical properties

The chemical properties of amylase afford an unusual opportunity for its quantification. Its molecular weight of 54,967\textsuperscript{12} enables it to be used as an antigen in an EIA sandwich assay; thus, it can be used to elicit an antibody response without having to be attached to a larger protein as a carrier. Hence, the opportunity for incorporating it into EIA procedures is possible. Pancreatic amylase and s-amylase differ by 7% in their amino acid sequence.\textsuperscript{8} This difference is only slight but is sufficient to differentiate the two isozymes by electrophoresis.\textsuperscript{10} Furthermore, production of specific monoclonal antibodies against each is becoming more successful.\textsuperscript{76,77,112,113-116,118}

Because amylase is an enzyme, its enzymatic reactivity has been utilized for assay procedures.\textsuperscript{12,67-69} Additionally, amylase's binding affords another opportunity for its determination. Robyt and French demonstrated that the active binding site of amylase binds a maximum of five glucose units. In addition, binding of the substrate appears to be polar with the reducing end closest to the catalytic group.\textsuperscript{131}
B. Cycloheptaamylose

The substrate/inhibitor binding characteristics of amylase were the basis of the development of this EIA since cycloheptaamylose is composed of seven glucose units in a circular structure. It is a perfect inhibitor of amylase, is inexpensive and is readily available. The unique properties of CHA enabled it to be used not only as the means of purifying p-amylase but also as a means of developing a unique sandwich assay. The principle of the assay is based on the CHA-amylase-antibody-HRPO complex being formed. The patient sample is added to a microwell precoated with CHA. The amylase (antigen/enzyme) binds to the CHA which is bound to the solid phase (microwell) during incubation, then later the excess amylase is washed off. The enzyme labeled antibody is allowed to incubate with the complex, then the excess labeled antibody is washed off. The indicator system consisting of a substrate and chromogen is added, and the color develops proportionally to the amount of amylase in the sample, as demonstrated in Figure 36.

Traditionally, in the heterogeneous noncompetitive enzyme immunoassay or sandwich assay, an antibody is coated onto the solid phase. The sample with the antigen is allowed to react, then the excess is washed off. A second antibody which is labeled with an enzyme attaches to a different site on the antigen. The excess enzyme labeled antibody is washed off, and the substrate and chromogen are added. The resulting color reaction from the addition of the substrate and chromogen is directly proportional to the amount of antigen in the sample.
Figure 36

Principle of the enzyme immunoassay for p-amylase or s-amylase
color development

Antibody-HRPO

Amylase

BSA-oxCHA

well

well + BSA-oxCHA + amylase + Antibody + color development

A + B

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In order to attach CHA to the microwell it was necessary to couple it to a protein first. The protein, in turn, was then adsorbed on to the surface of the polystyrene surface of the microwells. It was first necessary to convert at least one of the primary alcohols to a carboxylic acid and then, by an amide linkage, to couple it to the protein. Several means of oxidation of the CHA and its subsequent purification were attempted. The nitric acid mixture decomposed the DEAE cellulose column midway through its elution.\(^\text{127}\) A method to couple the CHA by using sodium periodate and dioxane to 6-aminohexanoic acid was also attempted. This would produce a compound which would act as a linker to the protein. This required the use of size exclusion chromatography utilizing Sephadex G-10-120. Likewise, coupling it to chloroacetic acid and then OVA and also BSA was also unsuccessful.\(^\text{131}\) Finally, the oxidation of a primary alcohol to a carboxylic acid was successful using the sodium nitrite-nitric acid mixture; however, care had to be taken, as the resulting brown nitrous dioxide gas is rather toxic. The resulting carboxyl group enabled CHA to be coupled by an amide linkage to BSA by first activating it with carbodiimmide resulting in an O-acylurea. Essential to this coupling was the presence of the NHS which helped to prevent the hydrolysis of the activated carboxylate of CHA. The first attempts at the BSA coupling were unsuccessful until NHS was added in the reaction mixture at sufficient strength.\(^\text{128}\) The BSA:CHA conjugate was then able to be coated onto the polystyrene surface of the microwells; thus, the enzymatic function of the antigen, amylase, resulted in its attachment to the well. The next part of the reaction...
depended on the antigenic properties of amylase with the utilization of the specific antibody against p-amylase or s-amylase.

C. Antibody production

1. Cell fusion

The first cell fusion gave the greatest yield of clones: 468! But this number of clones was very difficult to maintain for one person. A fusion resulting in 100 clones is an ideal number. Since newly formed hybridomas are highly unstable, it is probably a waste of time to screen the clones for positive antibody production for the first 10 days since some will either cease to be viable or stop antibody production. It was observed that only one-fourth of the cells from those initially picked remained viable and produced antibodies specific for the antigen. The maintenance of hybridomas is particularly tedious. Aseptic technique has to be maintained at all times. Initially, the incubator was washed with a 2% lysol solution and then rinsed with ethanol. The water that was kept in the bottom to maintain humidity was changed weekly. The outside of the flasks were rinsed with 70% ethanol prior to placing them in the hood for either transfer of cells or the addition of new media. Gloves were worn and washed with 70% ethanol before handling the flasks. Flasks were flamed prior to opening and again before entering with a sterile pipette or pouring. Even with all of these precautions, contamination did occur resulting in the loss of two cell lines. The fungal infection was the result of the addition of new media that was contaminated. It is advisable to never change all the cell flasks or wells of a growing cell line at the same time.
The initial freezing technique for maintaining cells proved unsuccessful; hence, the back up lines from the first fusions were lost. Initially, cells were placed at -20°C for 2 hrs, then -80°C for 4 hrs, then transferred to the gaseous phase of liquid nitrogen. Later, this was changed to transferring the cells immediately into gaseous phase of liquid nitrogen. The amount of DMSO was also reduced from 10% to 6% with 20% FBS in serum free media. Recovery of frozen cells was usually from 50 to 75%.

2. Ascites fluid production

Initially, ascites fluid production was only minimally successful. Other strains of mice that were tried were Balb/CJ and a "CF1"; both of these yielded ascites fluid only in one out of ten attempts. The mouse strain was changed to the Balb/CByJ, and the concentration and amount of cells injected were increased. It is also essential that the cells are in the log phase, not in the stationary phase, of growth when they are injected. Hybridoma cells are rather large and fragile; hence, they were drawn into the syringe without a needle. A 25 G needle was later placed on the syringe for injection. When a larger gauge needle was used, the cell solution tended to leak out of the peritoneal cavity. After the ascites fluid was produced, as was evident by the swelling of the abdomen of the mouse, it was also possible to recover hybridoma cells from the peritoneal cavity. The ascites fluid contained both hybridoma cells and fibroblast which eventually died. The media needed to be changed daily since contamination was at a greater risk with the ascites fluid due to the collection technique. Attempts at drawing the ascites fluid from the peritoneal cavity into a
syringe proved unsatisfactory. Invariably, the intestine or peritoneum would block the bevel of the needle. The Incomplete Freund’s Adjuvant, which gave slightly higher volume yield of ascites fluid, required 7-10 days inoculation prior to cell infusion. This was either advantageous if hybridomas were not in sufficient quantity for injection or disadvantageous because of the extra time required. Since sufficient antibody was purified from the pristane primed mice, the two highest ascites fluids produced were frozen but never extracted to determine the yield of antibody.

D. Diagnostic groups

The two groups with the highest incidence of acute pancreatitis are those patients with gallstones and alcoholism. The high incidence association with gallstones is often the result of the gallstone blocking the ampulla of Vaater, which is shared by the bile duct in some individuals. Other groups are also predisposed to producing a pancreatitis such as those individuals suffering from an infarction, ischemia, and cardiopulmonary bypass surgery (which also can have a hyperamylasemia without a pancreatitis). The administration of calcium chloride appears to be dose related with pancreatic cellular injury. This is a curious phenomena since both calcium and chloride are necessary for amylase activity. Ironically, surgical procedures in and around the pancreas, such as endoscopic retrograde pancreatography, that are often performed for treatment and/or diagnosing pancreatitis are known to elevate amylase levels and have also been known to induce a pancreatitis. The highest levels of amylase, greater than 1000 U/L, are seen in gallstone related pancreatitis.
Increased s-amylase levels frequently associated with cardiac surgery are often associated with the severity of pleural effusion.\textsuperscript{4} Total body irradiation also increases the quantity of serum s-amylase.\textsuperscript{24}

Reduced p-amylase is associated with chronic pancreatitis\textsuperscript{79} and carcinoma of the pancreas.\textsuperscript{66}

Renal failure is associated with an increase in both amylase isozymes.\textsuperscript{66}

Trauma to any organ of amylase predominance (parotid gland, submandibular gland and the pancreas) will cause an increase in the respective amylase levels.

\textbf{E. Human serum samples}

1. Sample diluents

Sample diluent was of primary concern in this EIA procedure since the determination of amylase not only depended on the antigenic properties of amylase but on its enzymatic properties as well. It is not surprising that chloride ions were necessary for the enzymatic reactivity of the amylase since this is a well documented fact.\textsuperscript{4} However, the necessity of placing the amylase standard in human serum to optimize the reaction was. Although high concentrations of the protein in human serum, as was seen in undiluted specimens, reduced the binding of amylase, it was necessary to optimize the reaction of the standards. This was evident by the increased activity of the standards in human serum. The patient samples, on the other hand, had to be diluted with the diluent by at least 1:4. A 1:5 dilution was chosen to reduce the interference of the serum protein in the reaction. This was advantageous in that the
sample size was reduced from 200 μL to 40 μL, with the remaining 160 μL being diluent.

2. Enzyme immunoassay of human serum samples

The isoamylase EIA, as it was performed on patient samples, was consistent with the admitting diagnosis of patients with "pancreatitis" that also showed increased amylase and lipase levels. In one case, the EIA showed an increase that the electrophoresis did not. Of the admitting diagnoses that were most probable to be correlated with increased s-amylase values: "beaten head and face", "gun shot wound neck", and "pneumonia", all showed increased s-amylase by electrophoresis and EIA. Unrelated admitting diagnoses such as "burns", "aneurism", "leukemia", and "flu" exhibited normal p-amylase and s-amylase levels by both methods. There were a few specimens that showed poor correlations. This was probably due to the fact that some patient samples were kept for a year at 4°C. Their amylase activity did not decrease; however, when they were tested by the EIA method, very high values were obtained for either p-amylase and/or s-amylase. This was probably the result of non specific binding of other serum constituents.

The source of p-amylase for the immunization and enzyme standards for the EIA procedure was normal pancreatic tissue. This is certainly a good source for isoamylase P₁ and P₂; however, it does preclude the detection of P₃ isoamylase. This was the case in three of the patient samples. P₃ isoamylase determined by cellulose acetate electrophoresis has a positive efficiency of 93% and a negative efficiency of 100%. Thus, the value of incorporating P₃ into an immunoassay procedure is worth
considering. However, it has not been reported that P₃ isoamylase is present without an increased total p-amylase; therefore, the main advantage of measuring P₃ isoamylase levels is specificity not sensitivity. Pancreatic amylase levels appear to be more sensitive to the diagnosis of pancreatitis than total amylase levels. A method for determining P₃ levels would be even more specific for acute pancreatitis.⁷⁹

3. Electrophoresis

Electrophoresis procedures which are currently used for determining the levels of specific isoenzymes for amylase are labor intensive, and only a few specimens can be analyzed at one time. A typical assay employs two standards and two controls; therefore, out of an eight sample strip, only four samples can be run at once. The turn-around-time for the assay is also long. The electrophoresis alone takes 1.5 hrs plus 1.5 hrs of incubation with the substrate-dye. Then the gels need to be cleared, dried and scanned. Also, interpretation of the results obtained by electrophoresis is very subjective. In scanning the strip, the placement on the scanner can greatly influence the results. Often during the run, the samples do not move in a straight line but migrated at an angle. It is necessary to visually check the bands to insure that the scanner is not at an end of one of the bands instead of the middle. The bands have to be compared manually to the standards in the run to interpret which peak is which isoenzyme. The scanner does not automatically interpret which peak is which isoenzyme. The P₃ band can be confused with the S₁ band particularly if the level of s-amylase is reduced. Thus, marking the results of the scan for the integration of the percentages of each isoenzyme is, again, a subjective determination. Values of the
amount of any isoenzyme can easily vary 20%. Scans of the electrophoresis of several patient's samples are illustrated in Figure 37.

E. Summary

Tests that utilize enzymatic activity were the first improvement on the early colorimetric methods. The introduction of fluorescent methodology offered increased sensitivity, but they had the problem of contamination and high backgrounds. The introduction of radioimmunoassays was a major advancement in clinical chemistry in its sensitivity and specificity. However, these had inherent problems that were not initially obvious. With increased concern about the environment and safety in the work place for employees, the use of radioactive isotopes fell into disfavor.

The development of polyclonal antibodies and EIA methods have made the possibility of detecting nanogram levels of a substance available. Enzyme immunoassay procedures have advantages over colorimetric test for sensitivity and specificity. They also have advantages over radioimmunoassays which require special storage precautions, have limited shelf-lives, and require disposal of radioactive waste. Many of the enzyme dependent assays have the disadvantage that there are outside sources of the enzyme in the indicator system. This is the case in tests employing the NAD+/NADH system. If a suitable blank is not part of the assay system, the high background can lead to erroneous results. Currently, the use of monoclonal antibodies adds the advantage of greater specificity. The old problems of hemolysis, lipemia, and bilirubin interference are not the concern that they once were.
Figure 37

Electrophoresis of human serum samples
A Patient 26, B Patient 208, C Patient 29, D Patient 201
Amylase determinations have proven to be a sensitive indicator for detecting pancreatitis. Pancreatic amylase determinations appear at present to be the most sensitive indicator of pancreatitis. The possibility of incorporating a monoclonal specific for P₃ isoamylase is worth pursuing. With the incorporation of EIA specific for p-amylase, the sensitivity is not only enhanced but specificity is also increased. Pancreatic amylase and s-amylase determinations have the possibility of being automated, thus easily incorporated into the clinical laboratory. Initially, the value of isoamylase EIA determination is in its specificity in detecting pancreatitis; however, it can also help in distinguishing a pancreatitis from other causes of increased amylase levels. Pancreatic amylase measurements by EIA becomes particularly advantageous with the need for a short turn-around-time. This p-amylase EIA method can be used as a diagnostic test with an assay time length of 2 hours under optimized conditions. This could probably be shortened to 1 hour with the advantage that it can be automated. Hospital laboratories do not perform isoamylase determinations as the electrophoresis method is too costly and time consuming. Instead, they are sent to a reference laboratory which further increases the turn-around-time. The equipment for performing automated EIA procedures is already present in most sizeable hospital laboratories. Therefore, the incorporation of isoamylase measurements by EIA is a feasible possibility for the near future.


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APPENDIX

SOLUTIONS

Complete medium (100 mL):

- deionized water 75 mL
- FBS (HI) 10 mL
- RPMI 1640 with glutamine (10X) 1.04 g
- HT (50X) 2 mL
- NaHCO₃ (7.5%) 2.2 mL
- P/S (100X) 1 mL

Serum free media (100 mL)

- deionized water 88 mL
- RPMI 1640 with glutamine (10X) 1.04 g
- NaHCO₃ (7.5%) 2.2 mL
- NaOH (1 N) 0.3 mL
- P/S (100X) 1.0 mL

The pH was adjusted on the above two media with 1 N HCl to 6.8-7.0; then brought to volume with sterile distilled water. Media was filtered through a 0.22 μm filter unit into sterile 100 mL bottles and stored in the refrigerator. When it was needed for use, it was warmed in a 37°C water bath.

2x IMDM (100 mL)

- Iscove’s medium powder 3.54 g
- NaHCO₃ 0.6048 g
- α-thioglycerol 2 μL
- P/S (100X) 4 mL

Methylcellulose

2 g methylcellulose + 50 mL distilled H₂O; autoclaved for 15 min at 15 psi
Polyethylene glycol (PEG)

1.5 g PEG is autoclaved for 15 min @ 15 psi; then 1.5 mL serum free medium was added when PEG was warm.

**Solution T (15 mL)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal Bovine Serum</td>
<td>10 mL</td>
</tr>
<tr>
<td>HAT (100X)</td>
<td>1 mL</td>
</tr>
<tr>
<td>LPS (lipopolysaccharide)</td>
<td>0.2 mL</td>
</tr>
<tr>
<td>(4 mg/mL serum free IMDM)</td>
<td></td>
</tr>
<tr>
<td>thymocytes</td>
<td>2 x 10^8</td>
</tr>
<tr>
<td>2X IMDM</td>
<td>1.4 mL</td>
</tr>
<tr>
<td>distilled water</td>
<td>2.4 mL</td>
</tr>
</tbody>
</table>

Thymocyte suspension

Thymocytes were obtained as previously described and stored in complete medium at 4°C in a concentration of 1 x 10^8 cells. For a clone in a 96 well dish, 250 μL of thymocyte suspension containing 10^4 - 10^5 cells/mL in complete medium were used. Microwells were fed with 25 μL/well of the thymocyte suspension at least once a week for growing clones.

**MC-IMDM**

Four grams of methylcellulose in 100 mL of sterile distilled water plus 100 mL sterile 2X IMDM. It may be stored at -20°C for up to 6 mo.

**Protein A elution buffer**

Dissolve 5.46 g citric acid monohydrate (52 mM) and 3.28 g Na_2HPO_4·7H_2O (24.5 mM) in deionized water to a final volume of 500 mL. Adjust pH to 3.0 with 1.0 N NH_4OH.

**Substrate solution**

The substrate solution (solution A) contained 2% (w/v) hydrogen peroxide in 0.1 M citric acid and 0.1 M sodium phosphate, pH 5.0. The solution was stored at 4°C.
Chromogen solution

The chromogen solution (solution B) was prepared by dissolving 20 mg 3, 3', 5, 5'-tetramethylbenzidine (TMB) completely in 1.0 mL DMSO and then adding, 10 mL of glycerol, 40 mL methanol and finally bringing it to 100 mL volume with distilled water to a final concentration of 0.2 mg/mL. The chromogen solution was stored at 4°C in a brown bottle.

Amylase screening solutions

Stock Substrate Solution

Dissolve 100 mg amylose (50 g/L) in 2 mL of dimethylsulfoxide by stirring at 80°C, in a water bath. Store at 4°C.

Working Substrate Solution

Mix 100 uL stock substrate solution in 4.9 mL 50 mM Tris buffer, pH 8.5. Make fresh when needed (1 g/L).

Iodine Stock

Dissolve 4 g KI in 50 mL deionized water. Dissolve 100 mg I₂. Bring to 100 mL with deionized water. Store in a plastic brown bottle at 4°C

Iodine Working Solution

Mix 20 mL stock iodine solution with 170 mL deionized water. Add 10 mL glacial acetic acid. Store in a plastic brown bottle at 4°C.