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Application of Monoclonal Antibodies Against Creatine Kinase MM and BB Isoenzymes for Creatine Kinase MB Isoenzyme Detection

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APPLICATION OF MONOCLONAL ANTIBODIES AGAINST CREATINE KINASE MM AND BB ISOENZYMES FOR CREATINE KINASE MB ISOENZYME DETECTION

by

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ABSTRACT

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Lei-Chieh Chuang
Old Dominion University, 1996
Director: Dr. James H. Yuan

Nondiagnostic ECGs and unknown characteristic chest pains increase the difficulty of Acute Myocardial Infarction (AMI) diagnosis. Biochemical markers, such as creatine kinase MB isoenzymes (CK-MB) and myoglobin, are employed for the early detection of AMI. Compared to myoglobin, CK-MB reveals a higher specific indicator for AMI detection owing to its abundance in cardiac tissue. Hence, a successful CK-MB detection is expected to improve AMI diagnosis and therapy.

A CK-MB detecting ELISA system was set up based on a sandwich assay principle by using specific CK-B and CK-M monoclonal antibodies. The dimeric nature of the CK-MB isoenzyme provides two epitopes on CK-B and CK-M subunits for antibody binding in the system. One anti-CK-BB hybridoma clone (B1D10) and three anti-CK-MM hybridoma clones (M1D6, M1G9, and M2B7) were obtained by the hybridoma technique.

Using Immulon I microwells, optimal performance of the CK-MB detecting ELISA system was achieved by using CK-B antibody as the solid phase immobilized

antibody, CK-M antibody as the second antibody conjugated with horseradish peroxidase, glutathione and BSA containing PBS buffer as the antigen incubation buffer, and BSA containing PBS buffer as the enzyme conjugate incubation buffer. A 12.3% CV for within-batch operation was determined for the system. A narrow CK-MB detection range, 7.3-15.0 ng.mL, was found in the current system, and this may be due to the deterioration of the purified CK-MB or the low affinity of the antibody. Furthermore, because 4.8% of cross-reactivity was observed with CK-MM, clinical validation was not performed in this study. Thus, this CK-MB detecting ELISA is successful in CK-MB detection but is not useful for AMI diagnostic detection.

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CHAPTER I

INTRODUCTION

A. Background

1. Characteristics of Creatine Kinase

Creatine kinase (CK; Adenosine 5′-triphosphate-creatine phosphotransferase, EC 2.7.3.2) is generally associated with the regeneration of ATP in contractile or transport systems and reversibly catalyzes the production of energy-rich creatine phosphate [1] (Fig. 1).

In vertebrates, ATP is the main energy usage form and creatine phosphate is the main energy storage molecule. Therefore, its predominate physiological function occurs in muscle cells and brain cells, which are rich in creatine phosphate. During a cycle of muscle contraction, metabolic process, or nerve signal transmission, a large amount of energy in the form of ATP is utilized. The phospho group of creatine phosphate is transferred to ADP to regenerate ATP and maintain the relatively constant cellular level of ATP, that is needed for proper biological function in cells. While the ATP level in cells is increased by the metabolic process, the phospho group is transferred from ATP back to creatine for energy storage as creatine phosphate. All of these processes are catalyzed by creatine kinase.

CK is composed with two freely dissociable subunits, each consisting of a single polypeptide chain containing no disulfide bridge [2]. Optical rotatory dispersion [3-6], tryptic digestion, and deuterium exchange studies indicate a compact globular

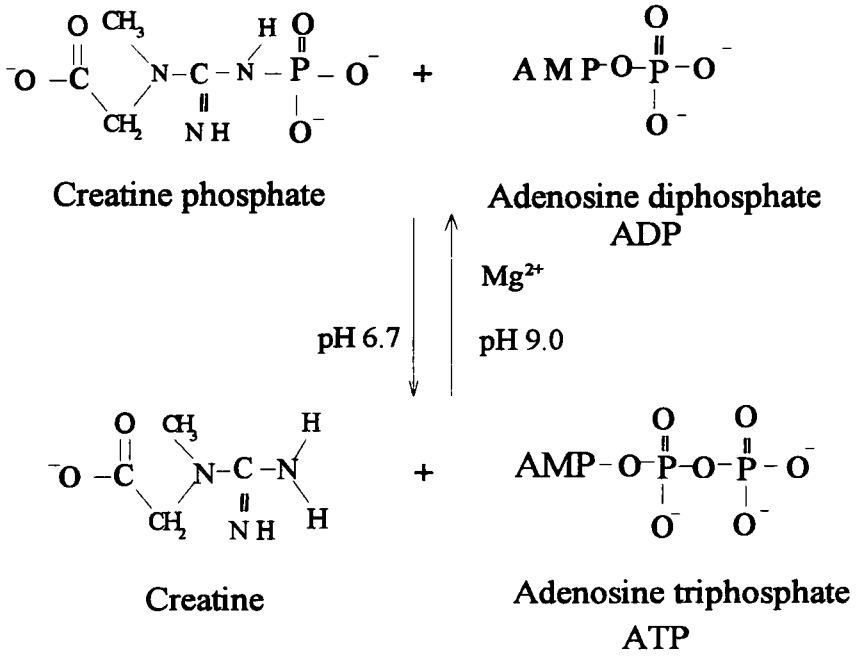


Fig. 1 Creatine Kinase Catalyzed Reaction
Creatine kinase catalyzes a reversible phosphotransfer reaction to maintain the ATP level in cells.

structure containing 25-30% α-helical and less than 15% β-pleated sheet conformation. By guanidinium chloride treatment [7], CK was dissociated without any apparent change in the structural organization, as measured by the frictional ratios (f/f_o) and the calculated axial ratios of subunits. Sedimentation studies under various conditions confirmed the calculated frictional ratio to be only 1.21 and the axial ratio to be 4.4 (for an assumed anhydrous prolate ellipsoid) [7]. Hence, the molecule can be envisaged as consisting of two compact cigar-shaped subunits lying side by side, rather than as two egg-shaped subunits joined end to end (Fig. 2). In vertebrate tissue, there are at least four subunit isoforms of CK based on a tissue-specificity [8-11]: two "cytosolic" CK-B and CK-M subunit isoforms (B: brain; M: muscle) [10] and two "mitochondrial" CK-Mia and CK-Mib subunit isoforms (Mi: mitochondria; "a" for the acidic and "b" for the basic isoforms; also called ubiquitous and sarcomeric CK-Mis, respectively) [11].

Both the CK-M and CK-B subunit isoforms are inactive as a monomer. Only the dimeric combinations of these two subunits can present enzyme activity [11]. Mitochondrial creatine kinase CK-Mia and CK-Mib subunits exhibit similar molecular weights to that of the CK-M subunit but fail to combine with either cytosolic CK-B or CK-M subunits. Therefore, there are three typical dimeric "cytosolic" creatine kinases, CK-MM (muscle type), CK-MB (hybrid), and CK-BB (brain type), *in vivo*, which have molecular weights of 85, 87, and 88.4 kDa, respectively [12].

In agarose gel electrophoretic separations [12], CK-BB was shown to migrate fastest toward the anode and hence is termed CK-1. This is followed by CK-MB

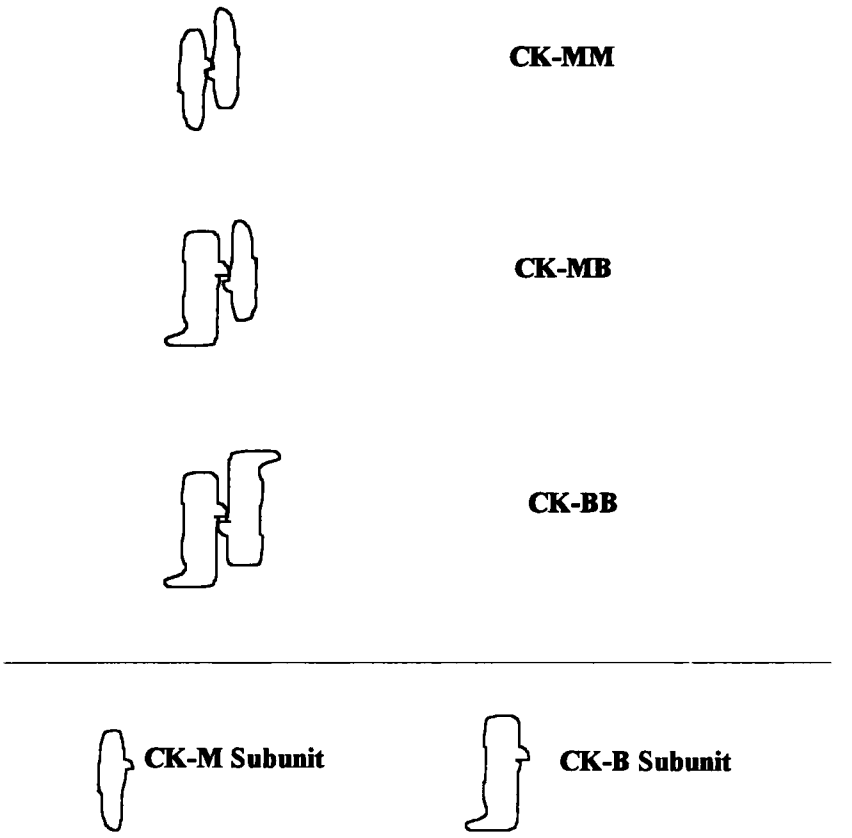


Fig. 2 Structure Schemes of Creatine Kinase
Creatine kinase CK-B and CK-M subunits showed anhydrous prolate ellipsoids with axial ratio of 4.4. The isoenzyme molecules can be imagined to be two compact side-by-side cigar-shaped subunits.

(CK-2) and CK-MM (CK-3), with the latter exhibiting the slowest mobility. In 1972, Smith [13] observed that the CK-MM and CK-MB isoenzymes can be fractionated espectively into "subtypes" or "isoforms" by isoelectric focusing. Both CK-B and CK-M subunits contain a C-terminal lysine residue, but only that of CK-M subunit can be hydrolyzed rapidly by carboxypeptidase, which is normally present in blood. On the basis of relative migration, CK-MM contains three isoforms, CK-MM1, CK-MM2, and CK-MM3, with only CK-MM3 found in tissues [14-16]. When CK-MM3 is released into circulation, it is irreversibly converted to CK-MM2 and then to CK-MM1, by successive enzymatic cleavage of a C-terminal lysine from each of the two CK-M subunits. Similar to the CK-MM isoforms, the CK-MB isoenzyme exists in two isoforms, CK-MB1 (post-synthetically modified upon release into the blood stream) and CK-MB2 (the tissue isoform) [14-18]. By high voltage electrophoresis [12], the CK-MM2 (one lysine residue removed), CK-MM1 (both lysine residues removed), and CK-MB1 (one lysine residue removed) in serum can be well separated. From the study of carboxypeptidase digestion [19], it showed that removal of the C-terminal dipeptide from rabbit CK-MM isoenzyme has no effect on catalytic activity. This implied that there is no activity difference between CK-MB1 and CK-MB2 or among CK-MM1, CK-MM2, and CK-MM3 isoforms. CK-B or CK-M subunits in all species are composed of a single polypeptide chain. Some studies [10, 19] revealed that CK-BB isoenzyme contains significantly less basic amino acids and more cysteine and aromatic residues than CK-MM isoenzyme; at the same time, the amino acid

composition of the CK-MB hybrid is between those of CK-MM and CK-BB isoenzymes. Therefore, this explains the order of electrophoretic mobilities of all CK isoenzymes.

Acid digestion, peptide mapping, and conventional fingerprints studies [10, 20-24] all indicated that CK-M subunits of different species have higher sequence homology than that between CK-M and CK-B subunits from a single species. Moreover, from SDS-polyacrylamide gel electrophoresis, Perryman and Strauss [25] reported that the CK-M subunits from different species have similar molecular weight. In contrast, the molecular weight of the CK-B subunit is different from that of the CK-M subunit and the molecular weight varies with each species. Cyanogen bromide cleavage studies [25] of CK isoforms from the different species showed that all CK-M subunits produce identical fragment patterns, while the cleavages of the CK-B subunits exhibit different patterns. In spite of the differences in sequences, molecular weights and cyanogen bromide cleavage patterns, the CK-B and CK-M subunits from different species contain homologous regions around an active thiol group and have retained the amino acid sequences essential for subunit interaction and enzymatic activity [19]. This indicates that the sequence difference between CK-B and CK-M subunits does not affect the enzyme function. The enzymatically active hybrid, CK-MB, will perform at different rates in dimer association, dissociation and kinetic properties because of the difference in CK-B subunits from different species.

From immunological evidence [26, 27], the tertiary organizations among the CK-BB or CK-MM isoenzymes from different species have been found to be more similar than those between the CK-MM and CK-BB isoenzymes from a single species. Hence, it is likely to find out the specific antibodies, which are raised only against one isoenzyme without cross-eractivity with the other isoenzyme from the same species. Although there may be some cross-reactivities among the specific antibodies with the same type of isoenzymes from different species.

Due to the heterogeneity between these subunits [12], the genes of CK-B and CK-M subunits were suggested and proven to be located on different chromosomes, 14 and 19, respectively. In 1986, the full length cDNA of human CK-M subunit was sequenced [28]. Subsequently, the cDNA clones of the human CK-B subunits from human brain and placenta libraries were isolated [29]. The entire coding region, the 3' untranslated regions, and 23 bp of the 5' untranslated region were sequenced for human CK-B subunit. A 77.77% nucleotide sequence homology exists between the coding regions of human CK-B subunit and human CK-M subunit. By contrast, no homology was found in the 3' untranslated region. This suggests that CK-B and CK-M subunits may have different translational regulation schemes.

During embroynic development [8, 19], the brain-type isoenzyme appears first in all human tissues. As development proceeds, the CK-BB level remains constant in the brain. During muscle cell differentiation *in vitro* or *in vivo*, the CK-BB isoform will transit to the hybrid CK-MB isoform, and then to the CK-MM homodimer. Hence,

in all of muscle fibers, the CK-MM isoform gradually increases to about 98%, with only 2% of CK existing as the CK-MB isoform. Whereas, cardiac tissue contains a significant quantity of CK-MB, corresponding to approximately 25-46% of all CK activity [12]. The myocardium is the only tissue from which CK-MB enters the serum in a significant quantity, and in other tissues, CK-MB is found only in small amounts.

The major CK isoeznyme is CK-MM (94-100%) in normal sera [30, 31], and CK-MB exhibits less than 4% of total CK activity. CK-BB is also present in trace amounts in the sera of healthy people. This pattern is thought to occur due to normal cell turnover or cell damage, since CK-MM is the major form of CK in most types of tissue. A variety of clinical conditions may cause tissues to release their cell contents, such as acute myocardial infarction (AMI). The infarction of cardiac blood vessle causes the ischemia of myocardiac tissue and the damaged cardiac tissue will release a large amounts of CK-MM and CK-MB isoenzymes into the circulatory system. This can alter the patterns of CK isoenzyme distribution in serum and provide valuable clinical information.

2. Detection of Acute Myocardial Infarction

Acute myocardial infarction (AMI) is one of the most prevalent life-threatening diseases in the world, secondary only to coronary arterial thrombosis. Annually, 750,000 individuals are admitted to United States hospitals diagnosed with an AMI, there is a 70% death rate for these patients [30]. Diagnosis of AMI must be made

quickly to allow for their treatment [31-37], which involves taking thrombolytic drugs or recanalizing the coronary arteries and reperfusing the ischemic cardiac muscle. These procedures must be initiated within 12 hours after the initial onset of the symptoms and preferably within 6 hours. The World Health Organization (WHO) has suggested the diagnosis of AMI to be based on: (1) the presentation of prolonged characteristic chest pain and/or abnormal electrocardiogram (ECG), and (2) the increased serum concentration of biochemical markers of AMI, for example the CK-MB isoenzyme. Many diseases of the heart will induce chest pain and nearly 50% of the patients with AMI initially exhibit nondiagnostic ECGs in Emergency Department presentation [30]. Therefore, it is critically important to get a sensitive biochemical marker for early diagnosis and verification of AMI in order to initiate an early and effective treatment.

Since 1962, many biochemical markers have been used for the determination of AMI [38]. Thus far, aspartate transaminase, myoglobin, lactate dehydrogenase (LD), troponins (T and I isoforms) and creatine kinase (CK-MM and CK-MB isoenzymes) have been explored for early diagnosis of AMI. Within the first 4-6 hours after AMI, the concentrations of these proteins will increase and then decrease due to the clearance of the circulation system [30, 31]. The major difficulty in using these biochemical markers to determine AMI is that the concentrations of these markers are dependent not only on physiology, but also on the time from the acute event. When the heart muscle is damaged because of ischemia, cell death is followed by the leakage of

cellular contents. The plasma concentration time-profile [31] of each cellular protein after a cardiac ischemic incident depends on the extent of the damage to heart tissue, the rate of leakage from the cells, and the rate of clearance from the circulation [39].

The early diagnosis of myocardial infarction by biochemical markers is generally based on some special characteristics of these special proteins. To be the marker in diagnosis, these proteins must be released rapidly from the injured heart tissue, have extremely low serum level under the basal condition, and they must be detected with high sensitivity and specificity. In the past, CK-MM isoenzyme was a useful biochemical marker and diagnosis kits for CK-MM detection had been developed. Myoglobin (17 kDa), a smaller protein among the biochemical markers of AMI in plasma, increases its concentration in plasma soon after the onset of AMI. The usage of CK-MM and myoglobin as AMI biochemical markers may lack specificity because of the high contents of these proteins in skeletal muscle and the rapid increase of their plasma levels during skeletal muscle injury. Troponins I and T isoforms, one type of regulatory protein in plasma, have increased specificity for myocardial injury but have long half-lives in plasma. Thus, they cannot distinguish between acute and chronic events. The CK-MB isoenzyme (86 kDa), a larger biochemical marker of AMI in plasma, increases after AMI event, has a very low basal concentration in plasma and high specificity for heart tissue damage and release characteristics during AMI. Hence, CK-MB isoenzyme is thought to be the best biochemical marker for early identification of AMI.

In demonstration of elevated levels of CK-MB [30,31], 4% of the total CK activity or 8 μg/L is considered to be a specific indicator of myocardial damage from AMI. During myocardial infarction [1, 31], the CK-MB level begins to rise within 4 to 8 hours, peaks at 12 to 24 hours, and returns to normal level between 48-72 hours.

Recent reports [42, 43] suggest that CK-MB isoenzyme has a clinical sensitivity of 75-80% and specificity of 90-95% in the diagnosis of AMI from all diagnosed AMI patients. These data suggest that if the sensitivity of the CK-MB measurement can be improved, the total amount of CK-MB isoenzyme will be the best early marker of AMI because of its cardiac tissue specificity.

A variety of procedures are available for measurement of total CK activity in the serum [44-48], such as electrophoresis, immunoinhibition, immunoprecipitation, and immunocapture. The normal ranges of CK-MB levels in plasma are expressed as an actual CK-MB activity of 3-5 U/L or total CK activity of 0-4%. Currently, electrophoresis is the most popular detection method and commercial systems are widely available for this purpose. Improvement of the procedure [31] has reduced the analysis time to under 1 hour with a mass or activity detecting sensitivity of 1 μg/L or 2 IU/L. However, current method is still not applicable for emergency room use, since sophisticated equipment such as a densitometer equipped with fluorescent measurement is required.

The recent trend in CK-MB analysis is to measure CK-MB mass rather than activity. Because CK isoenzymes are not stable proteins and the half-life of CK-MB,

10 hours both *in vivo* and *in vitro*, is so short that its activity decreases very fast with time [12]. Once an AMI occurs, the serum concentration of CK-MB begins to increase within the first six hours, and there is a further increase until the twelfth hour to reach its maximum value. As the time goes by, the activity of CK-MB is progressively harder to measure accurately in a blood sample. Hence, the detection of enzyme activity loses accuracy.

In a CK-MB detecting immunoassay, the specificity of the assay can be achieved by using two different antibodies, often monoclonal antibodies, directedly against the CK-B or CK-M subunits, or the CK-B and CK-M contact region. Generally, the results of CK-MB mass measurement correlate well with that of activity measurement [48-50, 52]. The upper limits of the linear ranges of an assay vary from 50 μg/L (Icon Assay, Hybritech Corp., San Diego, CA) to 500 μg /L (Magic Lite Assay, Ciba-Corning Diagnostics Corp., Medfield, MA). Overall, an assay like the Icon kit is suited for emergency room use, as it requires minimal and inexpensive equipment and only a 10 minutes assay time is needed.

In the coming decade, the immunological-based methods will continue to be the main focus for clinical biochemical research and development. If more sensitive and specific monoclonal antibodies can be produced, an improved immunoassay can be developed, which will improve the early AMI diagnosis after the onset of AMI symptoms.

3. The Hybridoma Monoclonal Antibody Technique

In 1975, Köhler and Milstein [52] developed a hybridoma technique that fused antibody-secreting cells and myeloma cells to produce a special fused cells, called hybridoma. Originally, each of antibody-secreting cells, spleen or plasma cells, isolated from an immunized animal, can produce a single immunoglobulin for one antigen but with definite life time. And NS-1 myeloma cells, a type of B-cell tumor, can not produce any intact antibodies but can proliferate indefinitely. After nonsecretor myeloma cells are fused with splenic lymphocytes, the hybrids demonstrate an ability to produce a single type of antibody (immunoglobulin), and proliferate indefinitely. These hybridomas can be maintained *in vitro* or *in vivo* and will continue to secret antibodies with a defined specificity. The antibodies produced by hybridomas are known as monoclonal antibodies.

In the last two decades, the hybridoma technique has been widely applied to numerous immunochemical problems. The monoclonal antibodies produced from hybridoma cells provide a specific binding capability, homogeneity, and unlimited antibody production. This technique has become a powerful tool in biomedical research and clinical diagnosis [53].

4. Enzyme Immunoassay Techniques

Recently, enzyme immunoassays are used widely in clinical laboratory because they are easy to use and inexpensive equipment is required. After employing

monoclonal antibodies, these assays have become highly sensitive and specific for analyzing proteins, haptens, and cell constituents of biological interest. These immunoassays are based on either homogeneous enzyme immunoassay or heterogeneous enzyme immunoassay principles [31, 54].

Homogeneous enzyme immunoassay is commonly known as enzyme multiplied immunoassay technique (EMIT). In this procedure, it is not necessary to separate each step. A defined amount of competitive antigens, which have been labeled with the color signal related enzymes, are applied to compete with target antigens to form antigen-antibody complexes. Once the enzyme labeled competitive antigens bind with antibodies, the active sites on enzymes are changed by this binding and can not accept their substrates to have color signal any more (Fig. 3). Assay signal is proportional to the concentration of target antigen. This technique is only applicable to detect substances of low molecular weight such as therapeutic drugs and steroid hormones in blood and urine. It is simple and fast but less sensitive (no less than 10^{-6} mole/L) than heterogeneous enzyme immunoassay, thus, is not practical for use in a clinical laboratory.

The heterogeneous enzyme immunoassay requires a solid phase to immobilize the antigen or antibody, and color signal related enzyme labeled antibodies to permit the quantitation of the antigen-antibody-enzyme complex or the antibody-antigen-antibody-enzyme complex. The heterogeneous enzyme immunoassay may be performed by either competitive or non-competitive methods.

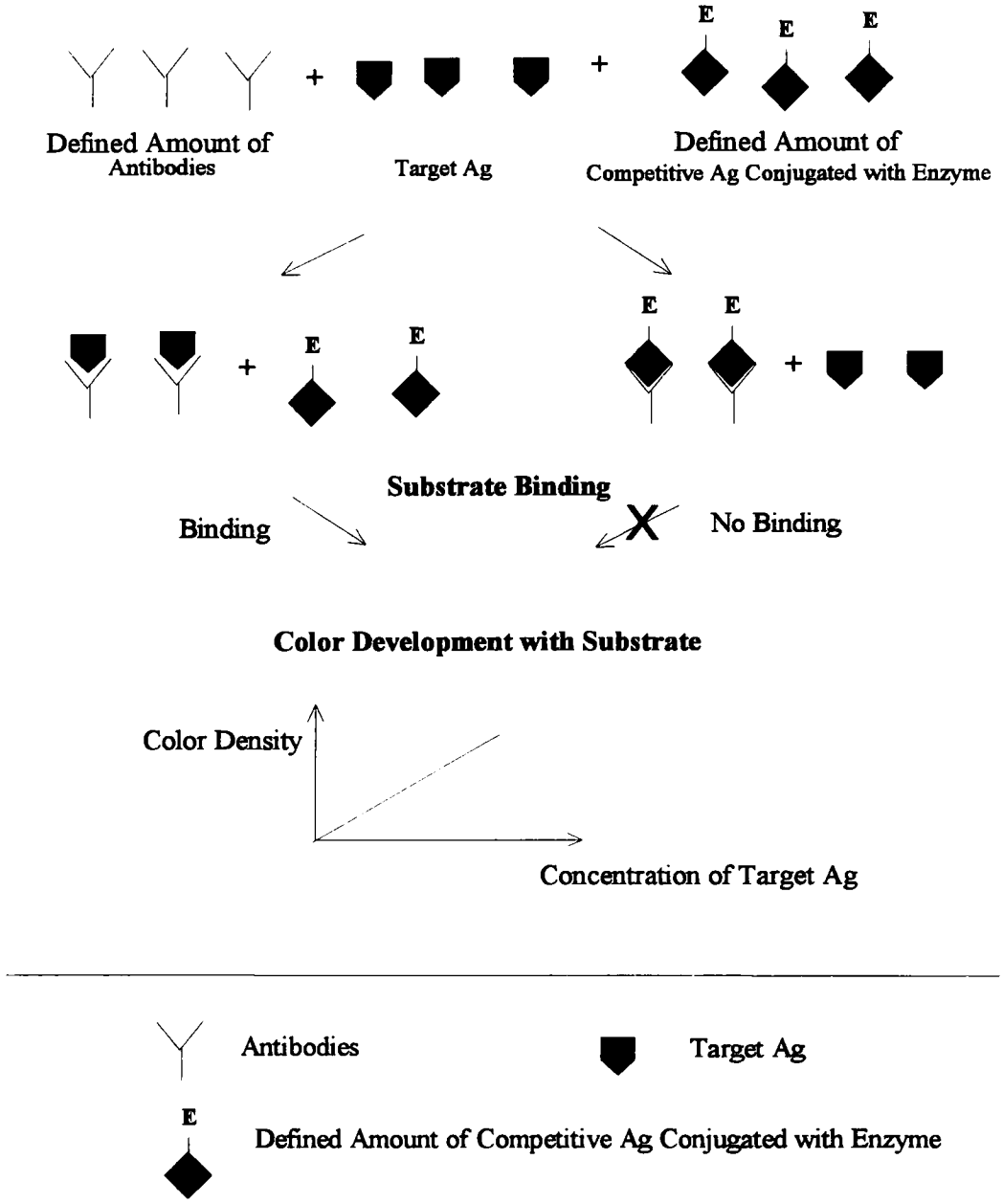


Fig. 3 Homogeneous Enzyme Immunoassay
This assay provides a simple procedure without separation step, since the assay of antigen-antibody-enzyme complex is performed directly in the reaction mixture. The assay signal is in propotional to the concentration of target antigen.

In competitive heterogeneous enzyme immunoassay, the target antigens are mixed with a defined quantity of color signal related enzyme labeled antigens. The target antigens and the labeled antigens compete for a limited number of antibody binding sites immobilized on the solid phase (Fig. 4). When the target antigens are absent, the highest amount of antibody-antigen-enzyme complexes can be obtained and the highest enzyme activity can be monitored by the color signal. But when a larger amount of target antigens are present, a smaller amount of enzyme labeled antigens can bind with the immobilized antibody, and a lower enzyme activity will be measured. Thus enzyme activity is inversely proportional to the amount of target antigens in the sample.

For the non-competitive heterogeneous enzyme assay, immunoenzymetric assays and the sandwich assay are two procedures commonly known. In the immunoenzymetric assay, the solution of target antigen is incubated with the color signal related enzyme labeled antibodies first. Then the rest amount of unbound enzyme labeled antibodies will be measured by binding with the antigens immobilized on a solid phase (Fig. 5). The enzyme activity observed is inversely proportional to the amount of antigen.

The sandwich type of heterogeneous enzyme immunoassay is also known as enzyme linked immunosorbent assay (ELISA). The target antigens are first bound to the primary specific antibodies, which have been immobilized on the solid phase. Then the antibody-target antigen complexes are bound by the secondary specific antibodies,

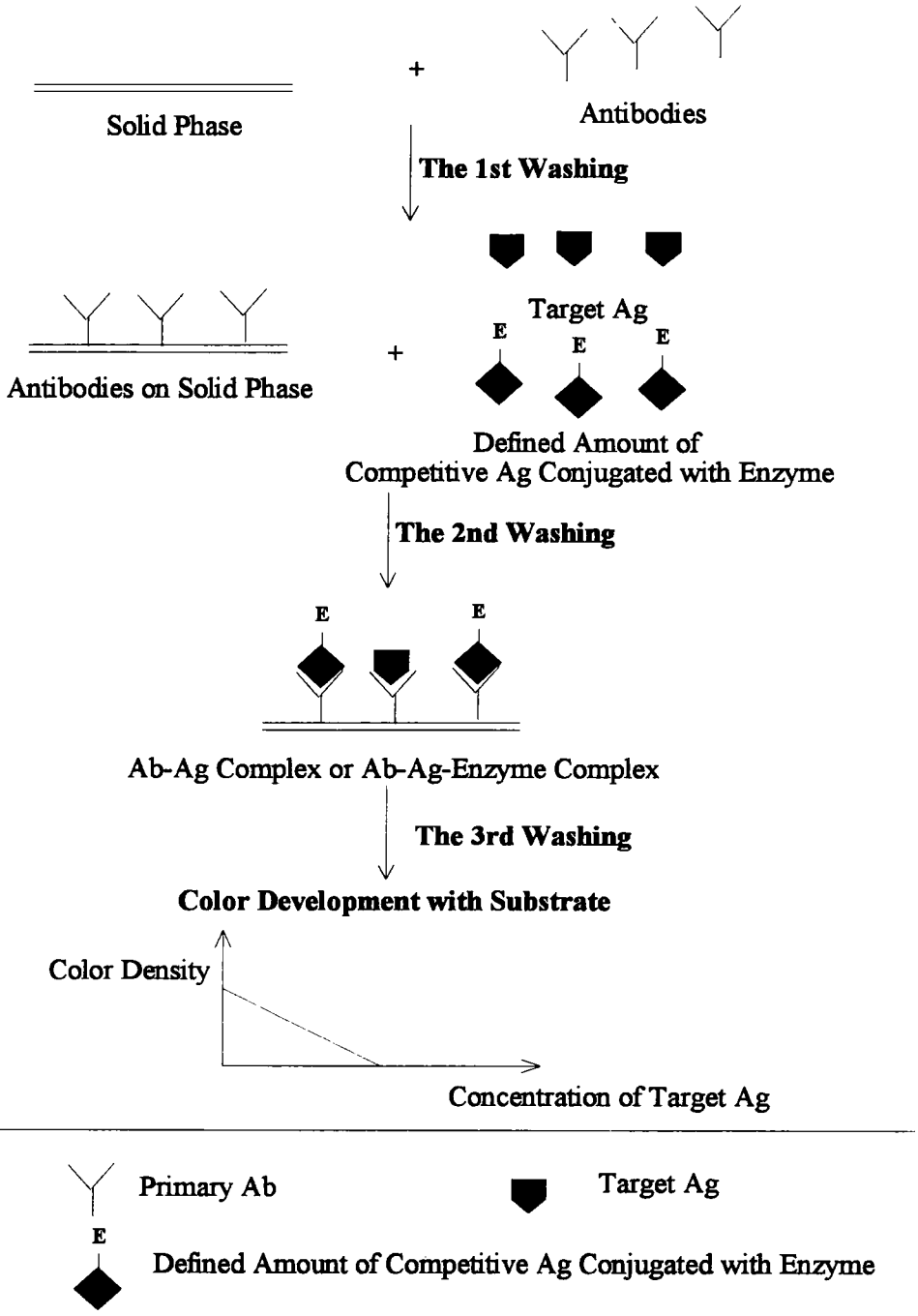


Fig. 4 Competitive Enzyme Immunoassay
A defined quantity of labeled antigens compete with the target antigens to bind with the immobilized antibodies. The assay signal is inversely propotional to the antigen concentration.

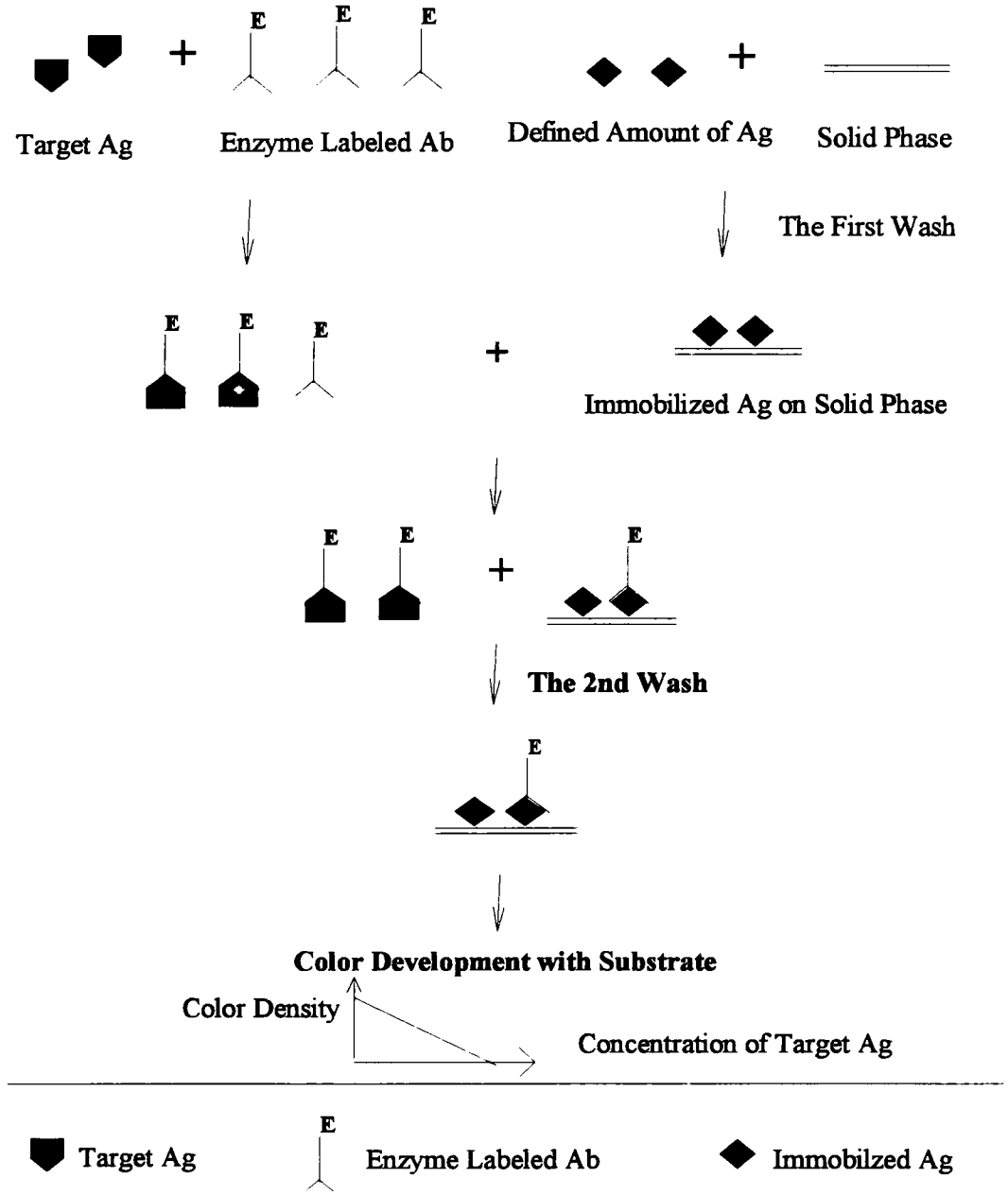


Fig. 5 Immunoenzymetric Assay
An indirected assay for detecting nonimmobilized antigen by reacting with a defined quantity of labeled antibodies. The remaining labeled antibodies reacts with the other immobilized antigen. The concentration of target antigen is inversely propotional to the assay signal.

which have been labeled with color signal related enzymes (Fig. 6). The enzyme activity observed by color signal is thus proportional to the amount of target antigens bound with the primary antibodies on the solid phase.

Among heterogeneous enzyme immunoassays, ELISA is the best assay to satisfy the detecting requirement, sensitivity and reproducibility. The requirements of this assay are two specific antibodies and target antigen possessing several epitopes for the binding with two specific antibodies. In CK-MB detecting ELISA system, there are more than two epitopes on CK-B and CK-M subunits for the binding with two different CK-B specific and CK-M specific antibodies, because CK-MB is a heterodimeric isoenzyme. Hence CK-MB detection could be easily performed by ELISA technique.

B. Objective

Due to the good serum mass-increasing timing and tendency after the onset of AMI symptom, CK-MB is expected to be an excellent biochemical marker for the early diagnosis of AMI. The goal of the proposed research is to produce one CK-M specific monoclonal antibody, and one CK-B specific monoclonal antibody, as well as develop an enzyme linked immnosorbent assay (ELISA) based on the sandwich principle for the quantification of CK-MB isoenzyme serum level.

CK-MM is the major component of CK in human serum and the content of CK-BB in human serum is normally very low. If CK-M antibodies are immobilized on

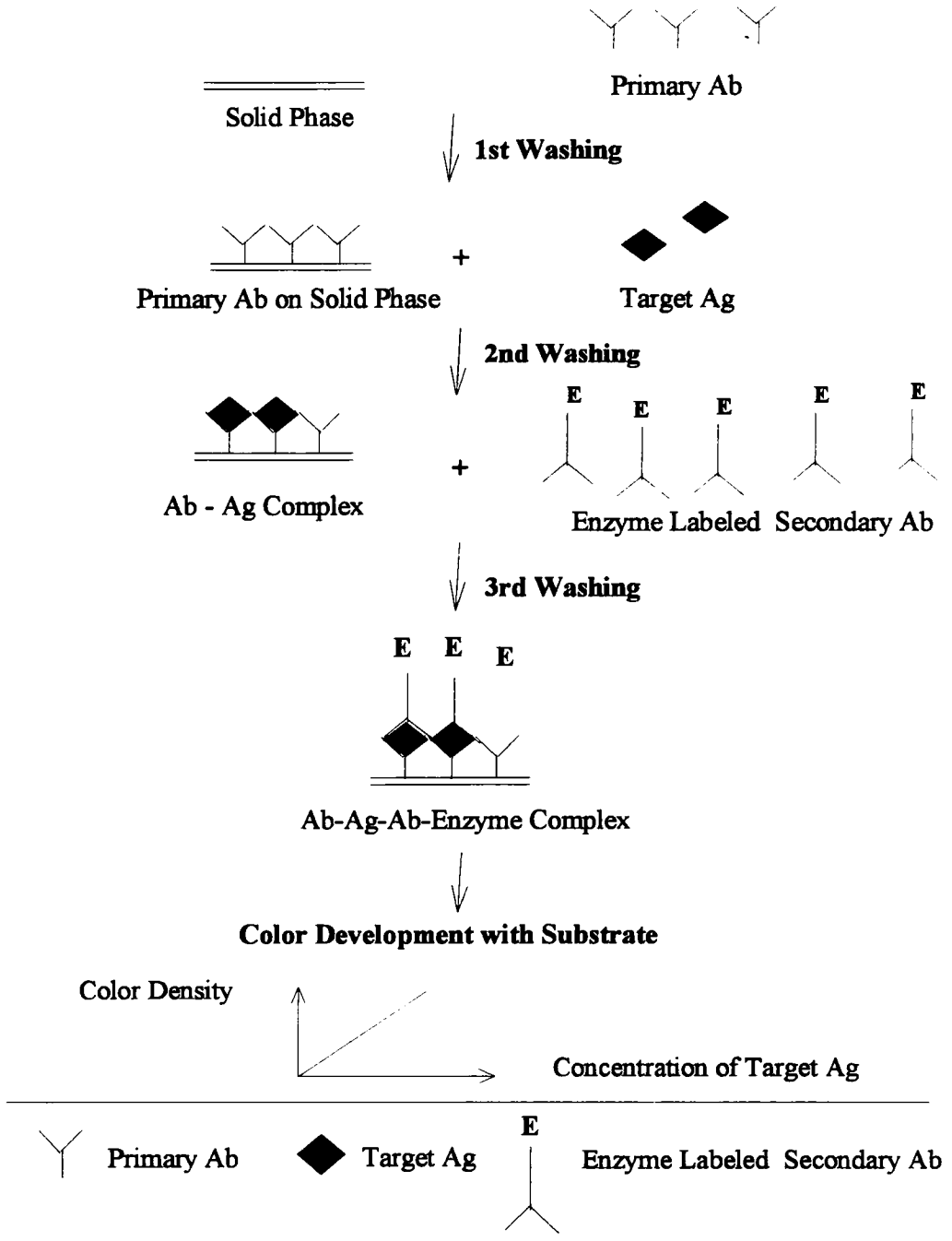


Fig. 6 Sandwich Enzyme Immunoassay (ELISA, Enzyme Linked Immunosorbent Assay)
The main assay was used in this research: A two-side binding assay for multiepitope antigen detection. The target antigen concentration is propotional to the assay signal.

the solid phase as the primary antibody, a large amount of CK-MM from human serum would interfere with the binding of CK-MB and decrease the sensitivity of the ELISA system. After choosing and coating a CK-B specific antibody on solid phase as the primary antibody, this CK-MM cross-reaction from human serum can be excluded completely and the specificity and sensitivity of the ELISA system can be improved.

Several types of solid phases have been applied to the heterogeneous enzyme immunoassays. The antibodies or antigens are immobilized by means of covalent linkages or simple adsorption [53] to solid supports such as latex particles [55], polystyrene beads [56], plastic tubes [57], nitrocellulose [58, 59], silicon rubber rod [60], magnetic particles [61], and microwells [62]. However, a simple physical adsorption to a plastic surface, such as polypropylene and polystyrene (tube, microtiter plate or microwell strip), provides a better immobilization. For this reason and also for reasons of practical convenience, it is commonly practical to perform enzyme immunoassays in polystyrene microtiter plates with 96 wells or removable microwell strips with 8 wells or 12 wells per strip in a well holder for which automatic photometric reading devices as well as automated washing machines are commercially available.

Thus, the principle of the ELISA system as applied here will involve the immobilization of a CK-B specific monoclonal antibody (CK-B mAb; the primary antibody) to the microwell. Subsequent to the immobilization of the primary antibody, the CK-MB containing sample is applied into this system to form the CK-B mAb –

CK-MB complex. After system washing, a horseradish peroxidase (HRPO) labeled secondary CK-M specific antibody (CK-M mAb-HRPO) is added to form a CK-B mAb – CK-MB – CK-M mAb-HRPO sandwich complex. Then the concentration of CK-MB isoenzyme can be determined by the colormetric assay of the HRPO enzyme activity. The order of development and specific objectives of this proposed research are as follows:

1. The isolation, purification, and identification of CK-MB isoenzyme from human cardiac tissue.
2. The generation of hybridoma clones specifically against CK-B and CK-M subunits by hybridoma technique.
3. The *in vivo* production of ascites fluids containing CK-B or CK-M monoclonal antibodies from mice.
4. The purification of the monoclonal antibody by a Protein A chromatography.
5. The conjugation reaction for labeling HRPO on CK-M specific monoclonal antibody (CK-M mAb-HRPO).
6. The development of a CK-MB detecting ELISA system on polystyrene microwells.
7. The performance study of the CK-MB detecting ELISA system. These will include a linearity study, a detection limit study, a precision study and a cross reactivity study.

The goal of this research is to produce CK-B and CK-M monoclonal antibodies and establish a good model for developing an CK-MB mass detecting ELISA system

by these two monoclonal antibodies. If high specificity and sensitivity are achieved, human serum samples could be tested and the correlation to the other commercial CK-MB detecting kits could be studied. This research is a preliminary study for development of a new diagnosis kit for AMI.

CHAPTER II

EXPERIMENTS

A. Materials: All of the chemicals are reagent grade

AAPER Alcohol and Chemical Co.: Ethanol (USP)

Allied Chemical: Dibasic sodium phosphate

American Type Culture Collection: NS-1 (non-screening mouse myeloma) cells

AMICON: Ultrafiltration system with YM-10 membrane, Centricon 3 microconcentrator.

Arthur H. Thomas Co.: Counting chamber, scientific apparatus.

A/S Nunc-Denmark: Nunc-immuno module microwell strips

Becton-Dickinson: 1 cc, 3 cc, 5 cc, and 10 cc Tuberculin syringe.

Bio-Rad: Affi-gel blue gels

CIBA-CORNING: Electrophoresis system of CK isoenzyme kit

Corning: 24-well Microwell plates, 8-well removable strips

Dynatech Laboratories, Inc.: Immulon I, Immulon II, and Immulon IV removawell strips.

E.M. Scienec: Sulfuric acid

Ethicon: 4-0 (1.5 metric) Silk

Falcon: 25 mL Culture flasks, 50 mL culture flasks, 10x35 mm Petri dishes, 15 x 100 mm Petri dishes.

Fisher Scientific Co.: Boric acid, citric acid monohydrate, disposable cell crapers, sterilized disposable pipettes (1, 5, 10 and 25 mL), glycerin , sodium acetate, sodium hydroxide, sodium meta periodate, and 35 x 10 mm suspension culture dishes.

General Chemical Division: Sodium phosphate dibasic crystal

Gibco: Fetal bovine serum (FBS), RPMI medium 1640, and Iscove's medium (IMDM).

Hamilton: Microsyringe (50 μL)

Jackson Laboratories: BALB/CByJ mice

J.T., Baker Chemical Co.: (Ethylenedinitrilo)tetraacetic acid dissodium salt

Kai: Miltex stainless steel sterile surgical blade

Mallinckrodt Chemicals Inc.: Sodium bicarbonate, ammonium chloride.

Millipore: 0.22 μm Filter unit with filling bell

Nunclon: 96 Microwell plates

Richfood: Instant and extra grade nonfat dry milk

Scientific Products Division: Microhematocrit capillary tubes SIP cover glass 22 x 22 mm

Scripps Laboratory: Creatine kinase-BB and creatine kinase-MM isoenzymes

Sigma: 2-Aminoethanol, bovine serum albumin (BSA), DEAE-Sephadex A-50 (Diethylaminoethyl-Sephadex) anion exchanger, Diagnostics kit for the quantitative and kinetic determination of creatine phosphokinase in serum

plasma at 340 nm, glutathione reduced form, goat anti-mouse IgG (whole molecule) peroxidase conjugate, goat anti-mouse IgG (F_{ab} specific), goat anti-mouse IgG (F_c specific) peroxidase conjugate, HAT selective supplement (100X), horse serum albumin, HT medium supplement (50X), hydrogen peroxide, lipopolysaccharide (LPS), monothiolglycerol, newborn calf serum, ovalbumin (OVA), penicillin/streptomycin (100X), pig serum albumin, polyoxyethylenesorbitan monolaurate (Tween 20), pristane (2, 6, 10, 14-tetramethyldecanoic acid), Protein A Sepharose 4B gels, RPMI 1640 medium, sodium azide, sodium phosphate monobasic anhydrous, sodium chloride, succinic anhydride, 3,3′, 5,5′-tetramethyl-benzidine (TMB), and Trizma base (tris[hydroxymethyl] aminomethane).

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

B. Equipment

Amicon: Filtration System model 12 and 52 with a YM10 membrane (25 mm and 100 mm)

Bausch & Lomb: Spectronic 1001 UV-VIS spectrophotometer.

Beckman: Model J2-21 centrifuge, Model TJ-6 centrifuge with Model TJ-R refrigeration unit, Model CDS-200 computing densitometer scanner.

Bio-Tek Instruments: Microplate reader EL307C

CIBA-Corning: Electrophoresis cell, microliter sample dispenser.

Damon/IEC Division: IEC clinical centrifuge

DIGITAL: Model 110 expended scale pH meter

E-C Apparatus: EC-400 power supply

Forma Scientific: Model 3326 water jacketed incubator

GILSON: Model FC-100 micro fractionator

Intrumentation Specialties Co.: Type 6 optical unit detector with 340 nm filter

ISCO: Model UA-5 Absorbance/Fluorescence monitor

Nikon: Type 102 phase contrast microscope, SMZ-1 stereoscopic microscope

Barnstead/Thermolyne: Type 48200 Rotomix

Sigmamotor: Model AL4 peristalic pump

Unitron: Mic-2306 invert microscope

YSI: Model 34 conductance-resistance meter, equipped with a YSI Model 3418 conductivity cell (K=0.1/cm)

C. Methods

1. Purification of Creatine Kinase MB Isoenzyme

(A) Preparation of DEAE-Sephadex A-50 ion exchange resins

Six grams of DEAE-Sephadex A-50 ion exchange resin was equilibrated in 50 mM Tris-Cl buffer, pH 7.5 containing 100 mM NaCl at 4°C with several changes of

the buffer. The equilibrated ion exchange resins were then packed to be a 2.1 cm x 24 cm ionic exchange column and washed with 200 mL of 50 mM Tris-Cl containing 5 mM 2-mercaptoethanol (column buffer).

(B) Preparation of cardiac tissues

Human cardiac tissue was obtained as a generous gift from Dr. Wolfinbarger (Lifenet tissues) and was excess tissue from cardiac valve processing. Processing of cardiac tissue was done in accordance with the Old Dominion University Bloodborne Pathogen Control Plan. Cardiac tissue, frozen at -70°C, was thawed in the refrigerator and rinsed in cold saline solution. The fatty tissue was removed and the muscle was cut into small pieces. Then 2 mL of ice-cold column buffer per gram of tissue was added and the tissue was homogenized for three 30-second bursts on ice, with a 1 minute interval. The homogenate was centrifugated at 10,000 x g at 4°C for 30 minutes. The supernatant was collected and filtered through four layers of cheesecloth to remove any remaining fatty material.

(C) Purification of creatine kinase MB isoenzyme by DEAE-Sephadex A-50 ion exchange chromatography

The conductivity of filtered supernatant (crude extract) was adjusted to be less than that of the column buffer by adding deionized water and was measured by a conductance-resistance meter (YSI Model 34). A final 15 mL volume of the crude extraction was applied to the column as well as the effluent was collected by 3-mL per tube in a GILSON FC-100 micro fractionator and the absorbance at 280 nm was

monitored by an ISCO absorbance/Fluorescence monitor. After the sample had merged into the resin, the column was washed with column buffer. When the absorbance reading of the effluent returned to baseline, a linear gradient elution, with 50 mM to 500 mM NaCl contained in total 250 mL column buffer, was applied to elute the CK isoenzymes.

(D) Purification of CK-MB by affinity chromatography

The CK-MB isoenzyme isolated from DEAE-Sephadex ion exchange chromatography was then applied to an Affi-Gel Blue affinity column (0.9 cm x 15 cm) which was pre-equilibrated in 200 mL of column buffer at 4°C for 2 hours. The absorbance of column effluent was monitored at 280 nm by an ISCO UA-5 monitor. After further washing with 200 mL of column buffer, the absorbance of the column effluent returned to the base line and the CK-MB isoenzyme started to be eluted with the 250 mM NaCl in column buffer. The fractions with CK activities were pooled, concentrated to a final volume of 1-2 mL by using an Amicon ultrafiltration cell with a YM-10 membrane in storage solution, mixed with an equal volume of 100% glycerol, and stored at -80°C in an aluminum foil covered tube.

(E) Quantitative determination of creatine kinase

The protein concentrations of the purified CK isoenzymes were assayed by the Bradford method [63] using BSA as a protein standard. For the assay, 100 μL of each creatine kinase samples and the standard protein solutions diluted in PBS were pipetted into small test tubes, and then 500 μL of Bradford reagent was added and

mixed. After 20 minutes, the absorbances of the samples and standards were measured at 595 nm. The concentrations of the samples were determined from a plot of BSA standards.

(F) Determination of creatine kinase enzyme activity

For the determination of creatine kinase activity, the Sigma Diagnostics kit [64] was utilized. The single assay vial of the kit was reconstituted with 3.0 mL deionized water and the contents was dissolved completely by gentle inversion of the vial. After 0.5 mL of assay solution was pipetted into a curette, the reaction was initiated by adding 100 μL of properly diluted creatine kinase sample solution, and the NADPH absorbance change at 340 nm was monitored from the 5th-10th minutes after the initiation of assay (ΔA_{340}). The whole assay reacts as follows:

$$\text{ADP} + \text{Phosphocreatine} \xrightarrow{\text{CK}} \text{ATP} + \text{Creatine}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{Hexokinase}} \text{ADP} + \text{Glucose-6-Phosphate}$$

$$\text{G-6-P} + \text{NADH} \xrightarrow{\text{Glucose-6-phosphate dehydrogenase}} \text{6-Phosphogluconate} + \text{NADPH}$$

The enzyme activity was calculated using the following equation.

$$A = \frac{(\Delta A_{340}) \times 0.6 \times 1000 \times TCF}{5 \times 6.22 \times 0.1}$$

TCF: Temperature Correction Factor, 1.37 for 22°C.

0.6: Total assay volume.

1000: Conversion from micromolar/mL to micromolar/L.

5: Conversion of $\Delta_{340}A$ per 5 min. to ΔA_{340} per min.

6.22: Micromolar absorptivity for NADPH at 340 nm.

0.1: Sample volume (mL)

(G) Identification of creatine kinase isoenzymes

For the identification of creatine kinase isoenzyme, a Ciba-Corning electrophoresis method was used. CK isoenzymes were separated by agarose electrophoresis at 90 volts for 20 minutes, and the electrophoretic pattern of the gel was developed by a series of enzyme coupling reactions and visualized on a UV light box or scanned with a Beckman model CDS-200 computing densitometric scanner. The enzyme coupling reactions are as following equations.

$$\text{ADP} + \text{Phosphocreatine} \xleftarrow{CK} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{D-Glucose} \xleftarrow{Hexokinase} \text{Glucose-6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} + \text{NAD} \xleftarrow{\text{Glucose-6-phosphate Dehydrogenase}} \text{6-Phosphogluconate} + \text{NADH (fluorescent)}$$

2. Preparation of Monoclonal Antibodies

All handling and care of mice was done in accordance with USDA and NIH guidelines.

(A) Intrasplenic injection

A five week old BALB/CByJ mouse was anesthetized with 0.2 mL of 60 mg/mL pentobarbital solution *via* I.P. injection (dose: 40-70 mg pentobarbital/kg body weight). The mouse was placed on its right side and its left side was washed with 70% ethanol. A 0.5 cm incision was made aseptically into left abdomen below the rib cage, exposing the spleen but leaving the peritoneum intact. The antigen, CK-MM or CK-

BB, was diluted from 20 μL of 2.0 mg/mL in PBS to a final volume of 100 μL by saline and injected into the spleen with a 1-mL syringe using a 30G1/2 needle. The skin was stitchered aseptically with ethicon silk 4.0.

The first and second boosters were administered on the 11th and 13th days after the initial immunization by injecting 20-40 μL of 2 mg/mL antigen in saline *via* the tail vein with a 1-mL syringe and a 30G1/2 needle.

On the fourteenth day after the initial intrasplenic injection, a small incision on the end of the tail vein of the immunized mouse was made. A hematocrit capillary tube was used to collect 10-20 μL of blood. The blood sample was centrifugated at 1,000 x g for 10 minutes at 4°C and a microsyringe was used to draw up the antiserum. The antiserum was diluted with PBS for the titer assay.

(B) Titer assay of antiserum

Immulon II microwells were coated with 200 μL of 50 ng/mL CK antigen at room temperature for 5 to 16 hours. The microwells were emptied, washed 7 times with PBS and deionized water, respectively, and then air dried for 10 minutes. Ovalbumin solution, 200 μL of 0.3% OVA, was added to each well and incubated for 2 hours at room temperature to block non-specific binding of other proteins on the microwells. The microwells were emptied, washed 7 times respectively with 400 mL of PBS and 400 mL of deionized water, and again air dried for 10 minutes.

Antiserum was diluted 100, 200, 400, 800, 1,600, 3,200, 6,400, 12,800, 25,600, 51,200, 102,400, and 204,800 folds by PBS and then 200 μL of each dilution was

added into each antigen-coated microwell and the wells were incubated at room temperature for 2 hours, emptied, and washed 7 times with PBS and deionized water. The wells were air dried for 10 minutes. Two hundred microliters of 5,000 folds diluted goat anti-mouse IgG-HRPO in PBS was added into each well and incubated for another hour at room temperature. The microwells were emptied, washed 7 times each with PBS and deionized water, and air dried. To each well, 100 μL of solution A and B were respectively added and the color was developed at room temperature. The color development was terminated by adding 50 μL of 2 N H_2SO_4, and the absorbances at 450 nm were read by a microplate reader.

(C) Cell fusion

When the titer of the antiserum was above 1,000-fold dilutions, cell fusion was initiated. In the process, 1×10^8 spleen cells, 2×10^7 - 5×10^7 NS-1 myeloma cells, and 2×10^8 thymocytes were prepared prior to the start of the fusion.

(1) Preparation of spleen cells

The immunized and boosted BALB/CByJ mouse was sacrificed, and the spleen was removed and rinsed by passing through the serum free media in three petri dishes. The spleen was left in the third petri dish and cut carefully with scissors into small pieces. The medium was removed with a 1-mL syringe, and the residual tissue was ground on a wire sieve. The residual ground tissue and the medium was pushed through the wire sieve to free the spleen cells. 10 μL of spleen cell solution was diluted into 4 mL of serum free medium and the free spleen cells were counted using a

counting chamber under Nikon type 102 microscope with a laboratory counter. The cells were centrifugated at 500 x g at room temperature. The old medium was removed and discarded, and 5 mL 0.85% NH_4Cl was added to dissolve red blood cells. The thymocytes were centrifugated at 500 x g for 5 minutes at room temperature. The supernatant was discarded and the spleen cells were again resuspended in 5 mL of serum free medium.

(2) Preparation of NS-1 cells

NS-1 cells, propagated to a total number between 2-5 x 10^7 cells, were harvested and resuspended in 5 mL of serum free medium. The ratio of the needed NS-1 cells to the immunized spleen cells was between 2:1 to 5:1.

(3) Preparation of thymocytes

A 5 week old BALB/CByJ male mouse was sacrificed by servical dislocation and sterilized by washing the chest with 70% ethanol. The chest cavity was opened, and the thymus was removed and rinsed through 3 petri dishes, which contains 3 mL of serum free medium in each petri dish, to remove as much blood as possible. The gland was cut with scissors carefully into small pieces in the serum free medium of the third petri dish. The medium was removed with a 1-mL syringe, and the residual tissue was ground on a wire sieve. Then both were pushed through the wire sieve to free the thymocytes. Cell counting was performed by diluting 10 μL of cell suspension into 4 mL of serum free medium. The thymocytes were spun down at 500 x g at room temperature for 5 minutes, and a total of 2 x 10^8 cells were resuspended in a solution

containing 10 mL of FCS, 2 mL of 50X HAT, 0.2 mL of LPS solution (4 mg of LPS/mL of 1X IMDM), and 1X IMDM to a final volume of 15 mL.

(4) Cell fusion

The spleen cell suspension was added to NS-1 cell suspension in a 50 mL centrifuge tube, and they were gently and thoroughly mixed. The cell mixture was centrifugated at 500 x g at room temperature for 5 minutes and the supernatant was removed.

Cell fusion was performed with the addition of 1 mL of 50% PEG solution followed by serum free medium to a total volume of 10 mL. The medium was removed by centrifugation at 500 x g for 5 minutes, the cell pellet was first suspended in 15 mL thymocyte solution, and the solution was then added to 25 mL of warm MC-IMDM solution. The cell suspension was then divided into several 35 mm petri dishes (about 1.5 mL/dish), and allowed to be incubated in 5% CO_2, at 37°C for approximate 2-3 weeks prior to clonal selection.

(D) Clone picking

Ten days after cell fusion, the petri dishes were checked. The clones which had a condensed center and a smooth-defined edge were picked out with a 50 μL pipette under Nikon SMZ-1 microscope. Each clone was cultured in 50 μL of complete medium containing 10^4-10^5 thymocytes in per mL of medium in a 96-well microplate. Once the clones grew fully enough (growing surface more than 1/2-2/3 of well surface), they were passaged into the wells of a 24-well microplate and then to 25 cm^2

cell culture flasks. In the process, the culture media containing secreted antibodies were saved for clone screening.

(E) Clone screening

A similar procedure of microwell coating and blocking to that of antiserum titer assay was utilized for clone screening. In order to achieve optimal results, 2, 4, and 6 hour antigen coating was studied. Fifty to one hundred microliters of culture medium from each clone or complete medium (negative control) were added to the antigen coated microwells, and PBS was then added to a final volume of 200 μL. These microwells were incubated for 2 hours at room temperature. Finally, the screening assay was carried out according to the titer assay procedure of antiserum.

(F) Antibody production *in vivo*

The positive clones were cultured to a total number of 2-4 x 10^7 cells, harvested, and resuspended in 1-2 mL of serum free medium. The prepared cell suspension was then injected into the abdominal cavity of an 8 week old or older BALB/CByJ mouse which had been primed with 0.5 mL of pristane 4 days earlier.

One to three weeks after the injection, ascites was harvested dropwise by puncturing the abdomen with an 18G1 needle. The harvested ascites was centrifugated at 1,000 x g for 10 minutes at room temperature and the supernatant was subjected to the purification of monoclonal antibody.

(G) Purification of monoclonal antibody

For the purification of monoclonal antibody, the ascitic supernatant was applied to a Protein A-Sepharose affinity column (0.5 cm x 5 cm) which had been equilibrated in PBS. The column was further washed with PBS until the absorbance at 280 nm returned to the baseline as monitored by an ISCO Absorbance/Fluorescence Monitor. The bound antibody was then eluted with a citrate phosphate buffer, pH 3.0 (52 mM citrate and 24.5 mM phosphate), and the pH of the effluent was quickly adjusted to 7.0 with 0.1 N NaOH . The purified antibody was then dialyzed overnight against PBS at 4°C with two changes of the buffer.

After dialysis, the antibody solution was concentrated to a final volume of 1-2 mL by Amicon ultrafiltration system with a YM-10 membrane. The protein concentration of the purified monoclonal antibody was determined by its absorbance at 280 nm using an extinction coefficient $E^{1\%}$ of 13.5 [64].

$$\text{Anti-CK IgG Concentration} = A_{280} \times \frac{1 \text{ mg/mL}}{1.35}$$

(H) Conjugation of antibody with horseradish peroxidase by periodate oxidation

Equal volumes of horseradish peroxidase (HRPO) solution, 10.0 mg/mL in 0.1 M sodium acetate buffer, pH 4.5, and freshly prepared 0.1 M sodium periodate (in deionized water) were mixed, and the mixture was allowed to stand at room

temperature for 5 minutes. The reaction mixture was dialyzed overnight at 4°C against 6 L of 10 mM sodium acetate buffer, pH 4.0 with two changes of the buffer.

For coupling the periodate oxidized HRPO to antibody, equal volumes of the periodate oxidized HRPO solution and the anti-CK IgG solution, 2.0 mg/mL in 0.10 M sodium carbonate buffer, pH 8.3, were mixed and the mixture stood at room temperature for 4 hours. Finally, one twentieth total mixture volume of a freshly prepared sodium borohydride (1.0 mg/mL in deionized water) was added to the mixture to a final concentration of 50 μg/mL. Again, the mixture was dialyzed overnight against 6 L of PBS at 4°C with two changes of the same buffer. The HRPO conjugated antibodies were aliquoted (100 μL) into vials, and stored at -80°C.

3. Assay Condition Study

(A) Preliminary study

(1) Saturation study

Two hundred microliters of diluted CK-B antibody solution were added into microwells in duplicate, and incubated at room temperature for 16 hours. The microwells were emptied, washed with 400 mL of PBS and 400 mL of deionized water seven times, respectively, and then air dried for 10 minutes. Goat anti-mouse IgG (whole molecule), 200 μL of 5,000-fold dilution in PBS, was added into each microwell and incubated at room temperature for 1 hour. After washing, the bound

HRPO activities were assayed by a procedure similar to that described for antiserum titer determination.

(2) Coating time study

The CK-B monoclonal antibody solution (B1D10), 200 μL/well, was added into microwells, and incubated for a time period varying from 2 to 20 hours. Then, the assay was performed following the standard procedure.

(3) Pairing study

CK-B antibody, B1D10, and three HRPO conjugated CK-M antibodies, M1D6-HRPO, M1G9-HRPO, and M2B7-HRPO, were used for the pairing study. For microwell coating, 200 μL of the B1D10 antibody solution was incubated in coating the microwells at room temperature for 16 hours. Then the assay was performed according to the standard procedure.

(4) Blocking agent study

BSA (Sigma A-4503), OVA, horse serum albumin, pig serum albumin, and skim milk were studied at different concentrations, 0.5%, 1%, and 3%; and varying incubation times for the assay.

(5) Washing Studies

PBS, PBS + 0.05% Tween, PBS + 0.1% Tween, and deionized water were studied as possible washing agents for the assay. The washing was performed in sets of 3/3, 5/5, and 7/7 using any washing combination of the above solutions followed by deionized water.

(B) Antigen study

(1) Incubation buffer study

PBS, or 2 mM 2-mercaptoethanol, 0.1% BSA, 0.5% BSA, 1% BSA, 1 mM reduced glutathione, 1 mM reduced glutathione + 0.1% BSA, 1 mM reduced glutathione + 0.5% BSA, and 1 mM reduced glutathione + 1% BSA in PBS were studied as the possible sample incubation buffers for CK-MB assay. The assay was conducted under the optimal conditions.

(2) Optimal incubation time

Linearity study of ELISA was performed by 25, 20, 15, 10, 5, 2.5, and 1.25 ng/mL of CK-MB in the optimal incubation buffer for 30, 60, and 90 minutes. All assays were proceeded with the optimal conditions.

(C) HRPO conjugated CK-M antibody study

(1) Optimal dilution fold study

In CK-MB bound Immulon I microwells, 200 μL of serially diluted HRPO conjugated CK-M antibody solutions, 250, 500, 1,000, 2,000, 5,000, 10,000, 15,000, 20,000, 25,000, and 30,000 folds, were added and incubated at room temperature for one hour. Then, the assay was completed following the standard procedure.

(2) Optimal incubation buffer study

PBS, 0.1% BSA, 0.5% BSA, 1% BSA, and 2% BSA were studied as possible incubation buffers for HRPO conjugated CK-M antibodies. The assay was completed following the standard procedure.

(3) Optimal incubation time study

A linearity study of ELISA was performed by the incubation of CK-MB at varying concentrations from 0 to 20 ng/mL. The optimally diluted HRPO conjugated CK-M antibody solution determined previously was added and incubated for 30, 60, and 90 minutes. All assays were performed using the optimized conditions.

(D) Post incubation washing study

Two washing agents, PBS and deionized water, at sets of different washing combinations, 3/3, 5/5, 7/7, and 10/10, were studied as the possible washing conditions for the post CK-MB incubation wash and the post HRPO conjugated CK-M antibody incubation wash. The purpose of these wash steps was to remove the unbound CK-MB or HRPO conjugated CK-M antibodies and buffer components as much as possible without interference with the assay reactions. The assay was performed following the optimized conditions.

(E) Color development time study

The assay was performed following the optimized conditions determined previously. At the last step of the assay, after adding solutions A (substrate) and B (chromagen), the color development time was varied from 1.5 to 15 minutes to determine the optimal developing time.

4. Performance Study of CK-MB ELISA System

(A) Linearity study

Standard CK-MB solutions, from 1 to 500 ng/mL, were used for the CK-MB linear detection range study. The assay was performed according to the optimized ELISA in order to obtain the linear detection range of CK-MB.

(B) Detection limit study

CK-MB standards, at various concentrations within the linear detection range, and 28 replicated microwells of non-CK-MB contained blank solutions were utilized for the detection limit study. The assay was performed under the optimized assay conditions. The results obtained were subjected to analysis according to the procedure recommended by the International Federation of Clinical Chemistry (IFCC) [31].

$$\text{Detecting Limit} = \text{Mean} \pm 2.6\ \text{SD}$$

(C) Precision study

Standard CK-MB solution with a concentration of 10 ng/mL, and within the linear range, was used for the precision study. The assay was performed in 24 replicates under the optimal ELISA conditions. At the same time, a standard curve was studied. The results obtained were subjected to mean and standard deviation calculation.

$$\text{Coefficient of Variant (\%)} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100\%$$

(D) Cross reaction study

The cross reaction study was carried out to compare the reactivities of the antibody to detect potential cross-reacting substances. CK-MM, CK-MB, CK-BB, myoglobin, and human serum albumin were analyzed at the same concentration used for this study. The assay was performed under the optimized standard procedure as previously described.

CHAPTER III

RESULTS

A. Preparation of CK-MB Isoenzyme from Human Cardiac Tissue

Human cardiac tissue, 24.81 g, was homogenized in column buffer (50mM Tris-Cl, pH 7.5 with 5mM 2-mercaptoethanol) and filtered; supernatant, 15 mL, was applied to a 2.1 cm x 24 cm DEAE Sephadex A-50 ion exchange column, which was pre-equilibrated with column buffer. The CK isoenzymes were eluted with a linear gradient, 50-500 mM NaCl in column buffer (Fig. 7). Three fractions with CK activity were found (Fig. 8A): Fraction 1 (tubes 1-14), Fraction 2 (tubes 18-66), and Faction 3 (tubes 94-101). Tubes containing each fraction of CK activity were pooled separately and concentrated to a final volume of 2 mL by an Amicon ultrafiltration system with a YM-10 membrane. The purity identification of CK isoenzyme in each fraction was determined by an agarose electrophoresis, and Fraction 2 was found to contain major CK-MB isoenzyme with minor contamination of CK-MM isoenzyme (Fig. 9A).

Fraction 2 was further purified by an 0.9 cm x 15 cm Affi-Gel Blue affinity column in column buffer (Fig. 10). CK isoenzyme was eluted from the column with 250 mM NaCl in column buffer. The major portion (90%) of the CK effluent was found to be CK-MB isoenzyme, with the remaining 10% to be CK-MM isoenzyme (Fig. 8, 9A). The minor CK-MM was the successfully removed by further DEAE

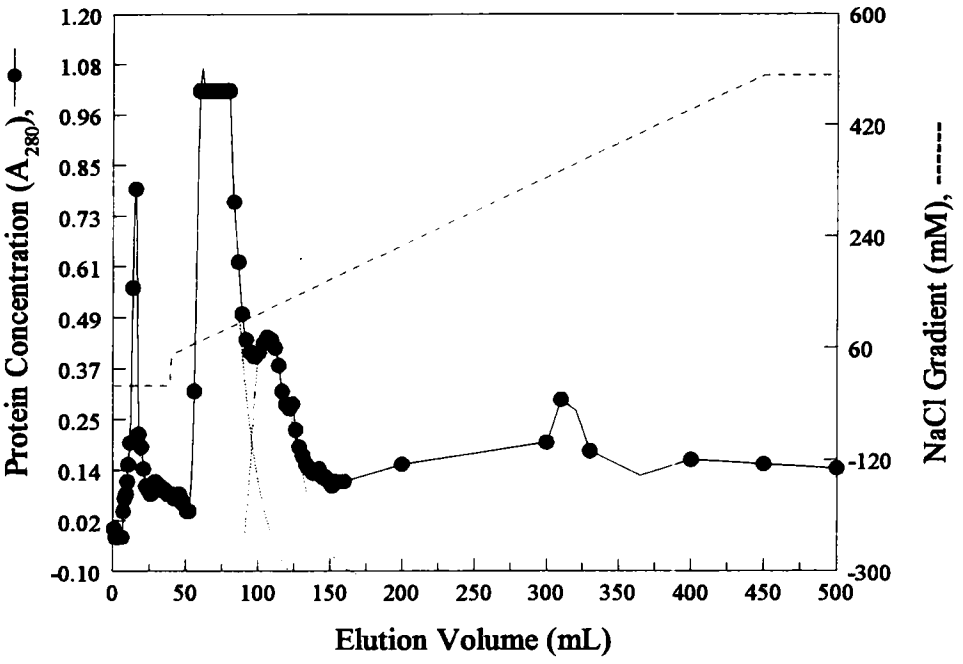
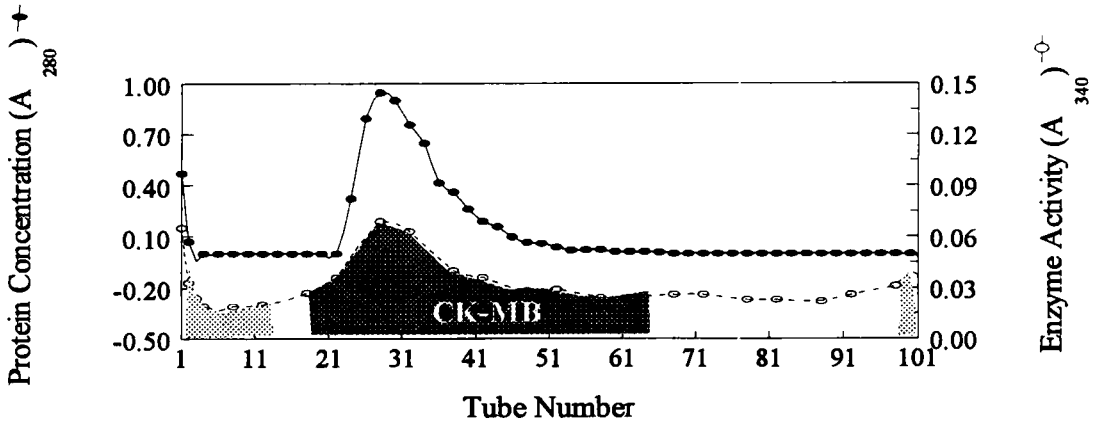


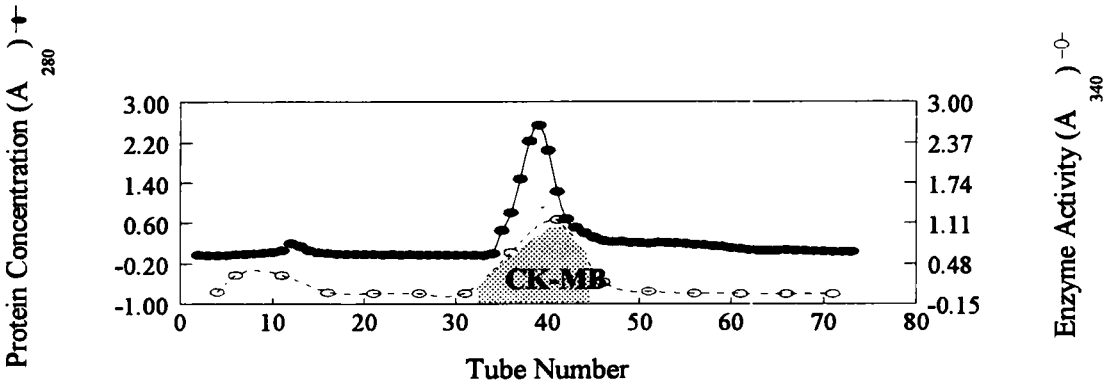
Fig. 7 Gradient Elution Profile of DEAE-Sephadex Ion Exchange Chromatography

After sample loading, 200 mL column buffer was used for column washing, 250 mL (125 mL of 50 mM NaCl contained column and 125 mL of 500 mM NaCl contained column buffer) gradient elution buffer was applied to elute the CK isoenzymes out and then the column was washed again by 400 mL of 500 mM NaCl included column buffer.

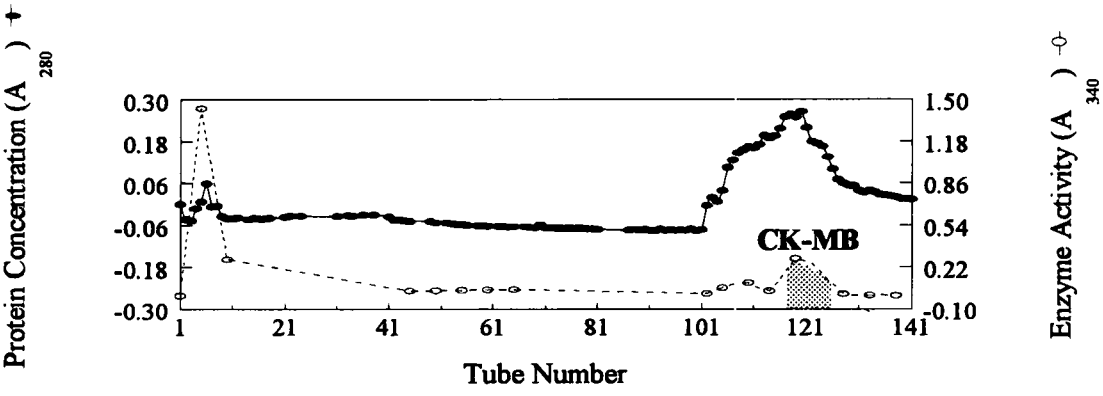
A.


Protein Concentration (A 280)
Enzyme Activity (A 340)
1.00
0.70
0.40
0.10
-0.20
-0.50
0.15
0.12
0.09
0.06
0.03
0.00
CK-MB
1
11
21
31
41
51
61
71
81
91
101
Tube Number

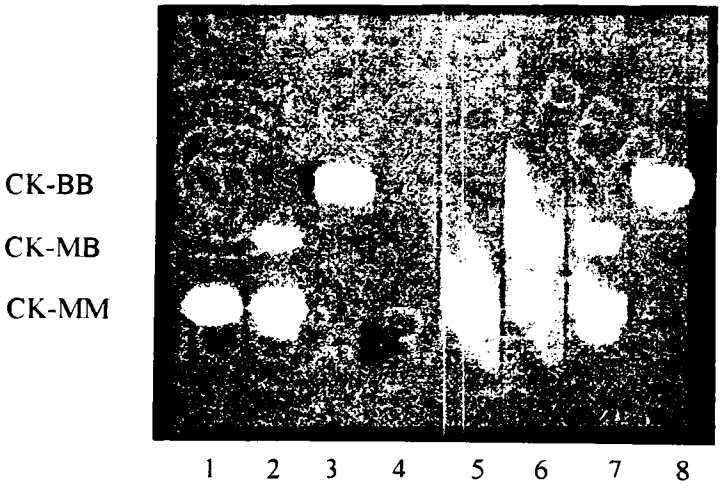
B.


Protein Concentration (A 280)
Enzyme Activity (A 340)
3.00
2.20
1.40
0.60
-0.20
-1.00
3.00
2.37
1.74
1.11
0.48
-0.15
CK-MB
0
10
20
30
40
50
60
70
80
Tube Number

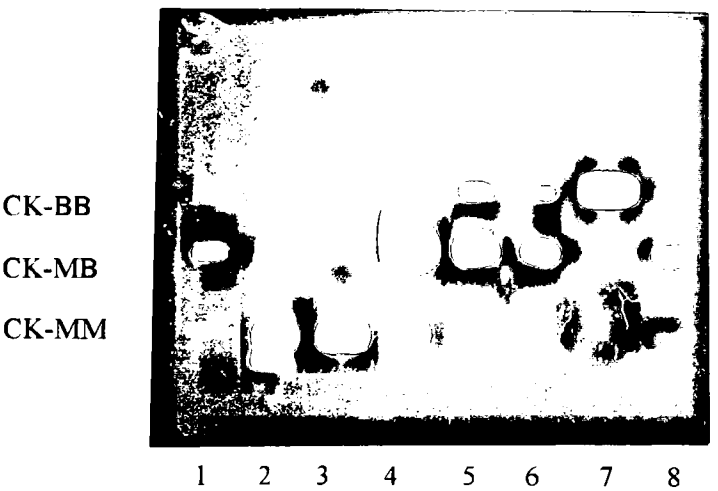
C.


Protein Concentration (A 280)
Enzyme Activity (A 340)
0.30
0.18
0.06
-0.06
-0.18
-0.30
1.50
1.18
0.86
0.54
0.22
-0.10
CK-MB
1
21
41
61
81
101
121
141
Tube Number

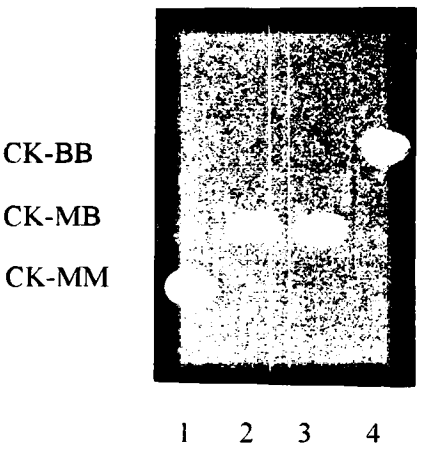
A.


CK-BB
CK-MB
CK-MM
1 2 3 4 5 6 7 8

B.


CK-BB
CK-MB
CK-MM
1 2 3 4 5 6 7 8

C.


CK-BB
CK-MB
CK-MM
1 2 3 4

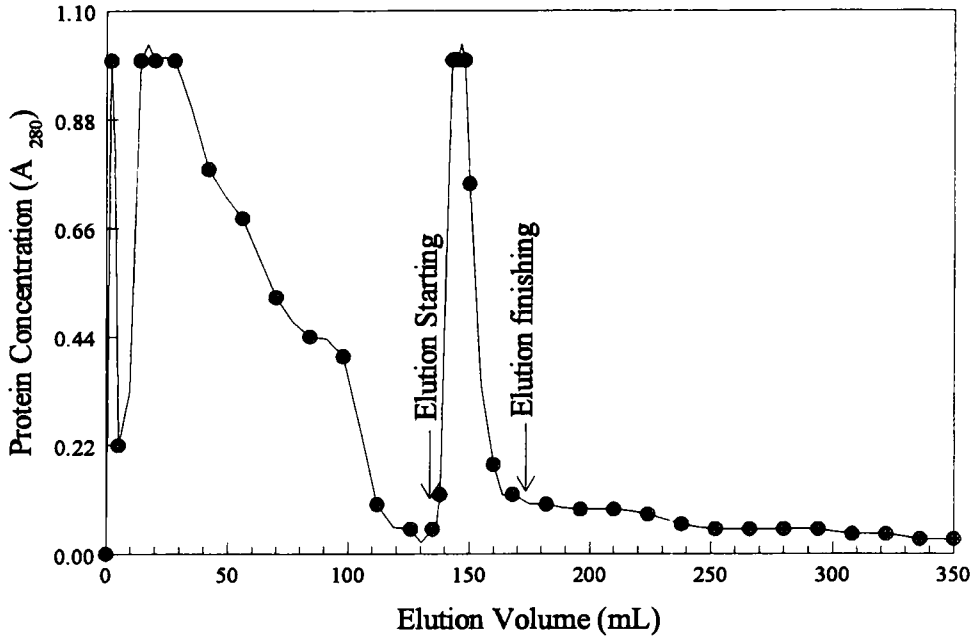


Fig. 10 Elution Profile of Affi-Gel Blue Affinity Chromatography
Affi-Gel Blue Affinity column was washed by 135 mL column buffer and then eluted with 250 mM NaCl included column buffer until the absorbance at 280 nm returned to baseline. The effluent was collected from the 135th-175th mL of effluent.

Sephadex ion exchange chromatograpy (Fig. 8). As determined by the agarose gel electrophoresis (Fig. 9C), 5.37 mg of pure CK-MB isoenzyme containing 1.908 IU of enzyme activity were obtained (Table 1). The purified CK-MB isoenzyme was concentrated to 1.74 mg/mL by an Amicon ultrafiltration system with a YM-10 membrane, and stored in 50% glycerol at -70°C until further use.

B. Preparation of Monoclonal Antibodies

1. Immunization of Mice

In mouse immunization, 40 μg of CK were given as the primary injection *via* intrasplenic route and another two boosters of 40 μg of CK were injected *via* tail vein. The titers of the first CK-BB and CK-MM immunized mice were checked at the 13th day after intrasplenic immunizations. The titer of the second CK-BB immunized mouse was checked at the 14th day. Using antibody-capture solid phase ELISA, the titers of the first CK-BB and CK-MM immunized mice were determined to be 3,100 and 3,200 (Fig. 11) respectively, and the titer of the second CK-BB immunized mouse was 1,200 (Table 1).

2. Cell Fusion

From the first cell fusion, 101 anti-CK-BB hybridoma clones were picked, and 13 of them were proven to be positive. Initially they all grew well, but later some of them started dying because the L-glutamine in the medium degraded quickly and no supplemental L-glutamine was added before running out, only one positive clone

Table 1 Purification of Creatine Kinase MB Isoenzyme from Human Cardiac Tissue

Human Heart: 24.81g

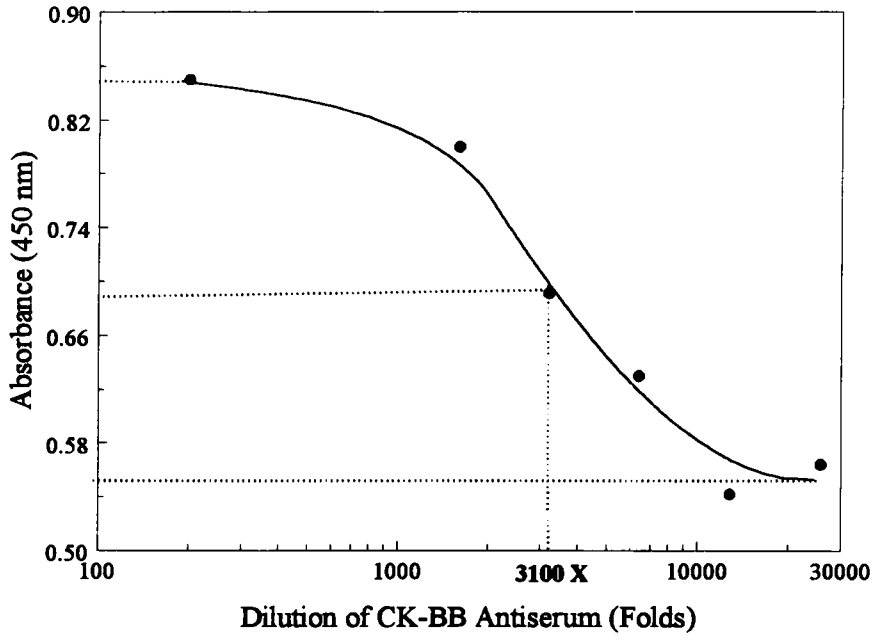
Fraction	Total Protein(mg)	Total Activity[3](IU)	Specific Activity(IU/mg)
The 1st DEAE Ion Exchange Column (Fraction 18-66 has been passed through Affini-Gel Blue Affinity Column)			
Fraction 1	192.57[1]	658.84	3.420
Fraction 2	20.70[1]	14.80	0.714
Fraction 3	18.07[1]	655.07	36.250
The 2nd DEAE Ion Exchange Column (from Fraction 2 Fraction)			
Fraction 1	30.40[2]	0.552	0.018
Fraction 2	4.68[2]	0.700	0.150
Fraction 3	0.25[2]	0.234	0.944
The 3rd DEAE Ion Exchange Column (from Fraction 1 Fraction)			
CK-MB	5.37[2]	1.908	0.355

1. Protein Concentration is the result of the calculation, Concentration = A_{280} x 0.8 mg/mL.

2. Protein Concentration is the result of Bradford Assay.

3. Enzyme Activity was decided by Sigma CPK Diagnostics Kit (METHOD 1.(F)).

A.



B.

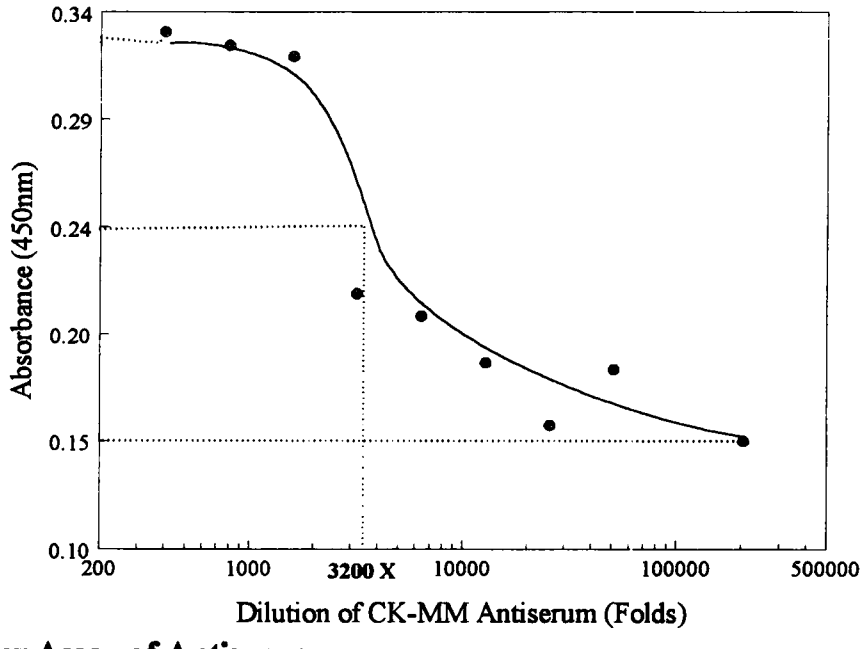


Fig. 11 Titer Assay of Antiserum

A. CK-BB Antiserum from 200 to 25,000 folds dilution were incubated and the absorbance at 450 nm was measured. A 3,100-fold dilution was obtained for the optimal titer. **B.** CK-MM Antiserum from 400 to 200,000 folds dilution were incubated and the absorbance at 450 nm was measured. A 3,200-fold dilution was obtained for the optimal titer. Experiment data points represent the mean of duplicate samples.

(B1F11) survived. However, this clone had lower CK-BB affinity than some of other clones that were lost. In the second cell fusion, 65 anti-CK-BB clones were picked, and 10 stable clones were obtained; however, only one, B1D10, had a high CK-BB affinity (Table 2).

The anti-CK-MM clones met the same situation as the anti-CK-BB clones because the characteristics of medium were not completely understood. Three positive anti-CK-MM hybridoma clones survived from the 188 picked clones: M1D6, M1G9, and M2B7 (Table 2). They were all found to have good specificities.

3. Clone Screening

Because CK-BB and CK-MM are very unstable proteins [12], the screening conditions of anti-CK-MM and anti-CK-BB clones were modified to optimize the results (Fig.12). Each Immulon II microwell was coated with 200 μL of 50 ng/mL CK-BB or CK-MM in coating solution for 4 hours at room temperature. The microwells were emptied, washed with PBS and deionized water seven times, respectively, and air dried for 10 minutes. Then, the microwells were blocked with 1% (w/v) BSA in coating solution at room temperature for 30 minutes.

From the results of screening, B1F11 was concluded to be a nonspecific CK-BB antibody and highly cross-reactive with CK-MM (Fig.13). Therefore, a second cell fusion for anti-CK-BB hybridoma clones was performed. Another CK-B antibody, B1D10, was proven to be a more specific antibody than B1F11 and have little or no

Table 2 Summary of Cell Fusion

Antigen	CK-BB	CK-MM	CK-BB
Intraplenic Injection (μg)	40	40	40
Boost (μg)	40	40	40
Titer	3100	3200	1200
Picked Clones	101	188	65
Positive Screened Clones	13	31	10
Growing Stablely Clones	1	3	1
Clone Name	B1F11	M1D6	B1D10
		M1G9	
		M2B7	

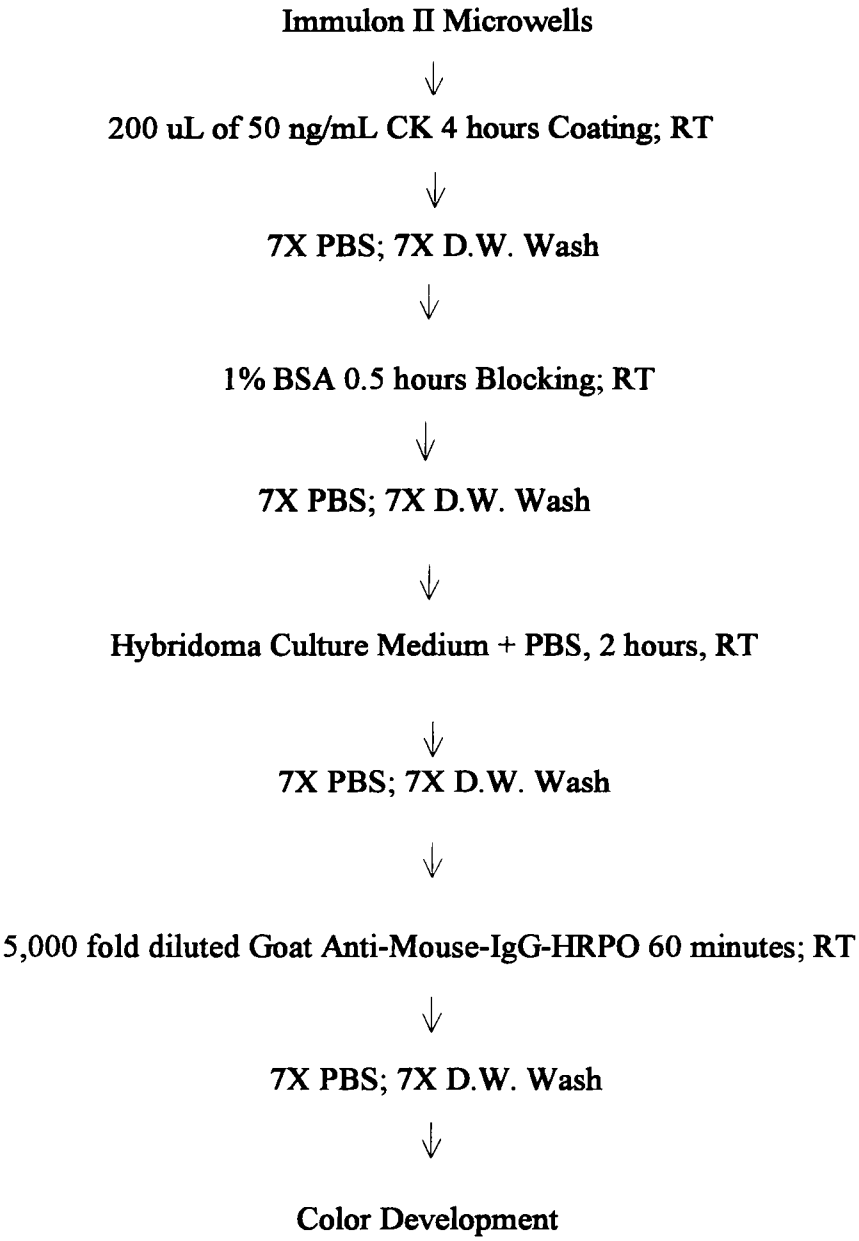


Fig. 12 Summary of Screening Assay
An antibody-capture solid phase assay was applied on Immulon II microwells.

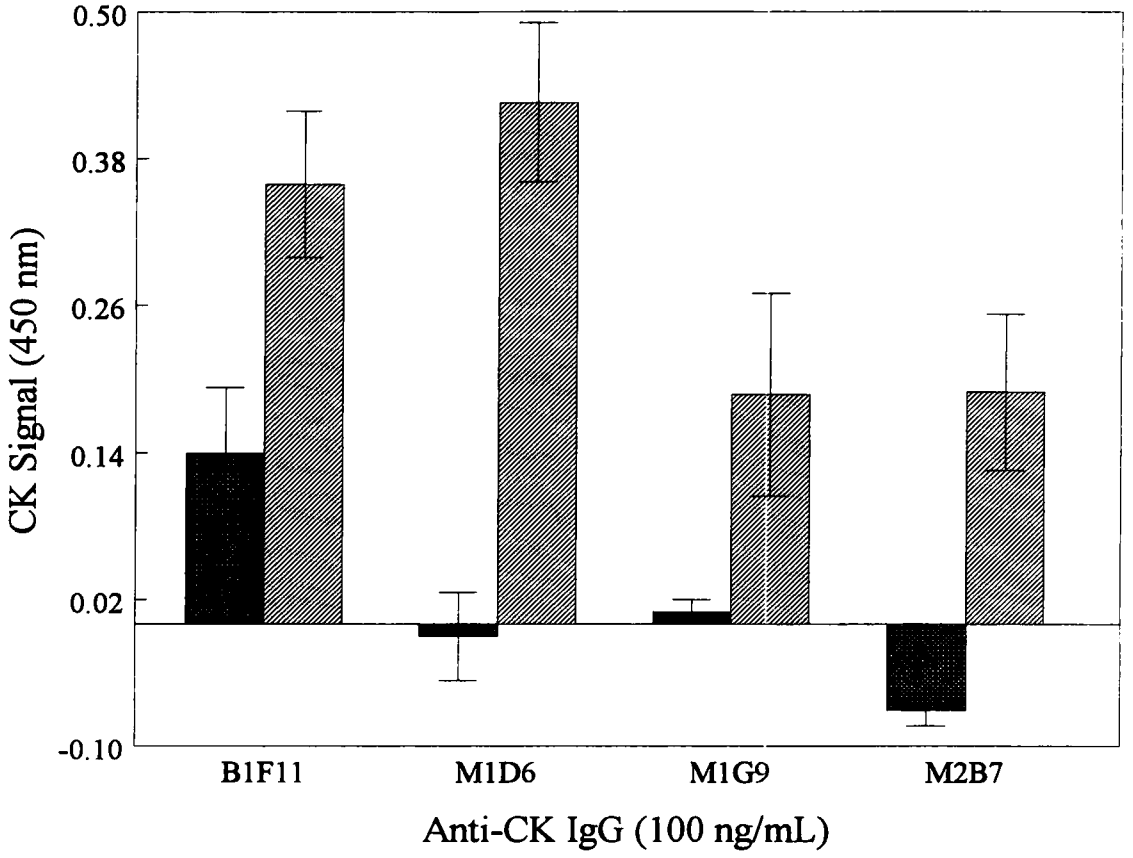


Fig. 13 Double Check of CK Antibodies

CK-B antibody (B1F11) and CK-M antibodies (M1D6, M1G9, and M2B7), 200 μL of 100 ng/mL, were incubated in CK-BB and CK-MM coated Immulon II microwells. All assay signals were the absorbance differences at 450 nm between antigens and blank.

CK-BB

CK-MM

cross-reaction with CK-MM and myoglobin (Fig. 14). Hence, it was expected to be a good CK-B antibody.

From the double screening of CK-M antibodies (Fig. 13), M1D6, M1G9, and M2B7 were screened in CK-BB and CK-MM coated microwells and all showed good CK-MM affinities. Among them, M1D6 has a better affinity than M1G9 and M2B7, as well as all of them had good CK-MM specificities.

4. Production of CK-M and CK-B Antibodies *in vivo*

Ascites production varied from 9 to 25 days by the intraperitoneal injection of 2-4 x 10^7 hybridoma cells with an average of 17 days. The total volume collected ranged from 0 to 7 mL. The total IgG from each mouse ranged from 0.75 to 3.09 mg/mL (Table 3). The average yield of antibody from ascites was 0.48 mg/mL (Table 3). Some clones, such as M1G9 clone, would form a large solid tumor rather than produce ascitic fluid suspension. After the death of the mouse, the abdominal cavity was opened and washed with saline; the large solid tumor was removed, cut into small pieces, and extracted with saline. Averagely, 2.33 mg of IgG were harvested by this solid tumor extraction (Table 3).

5. Purification of Antibodies

All antibodies from the culture media or ascites were purified by a Protein A-sepharose affinity chromatography (Fig. 15). Generally, ascites production *in vivo* was believed to be a better method for antibody production [53]. Because only 5% of

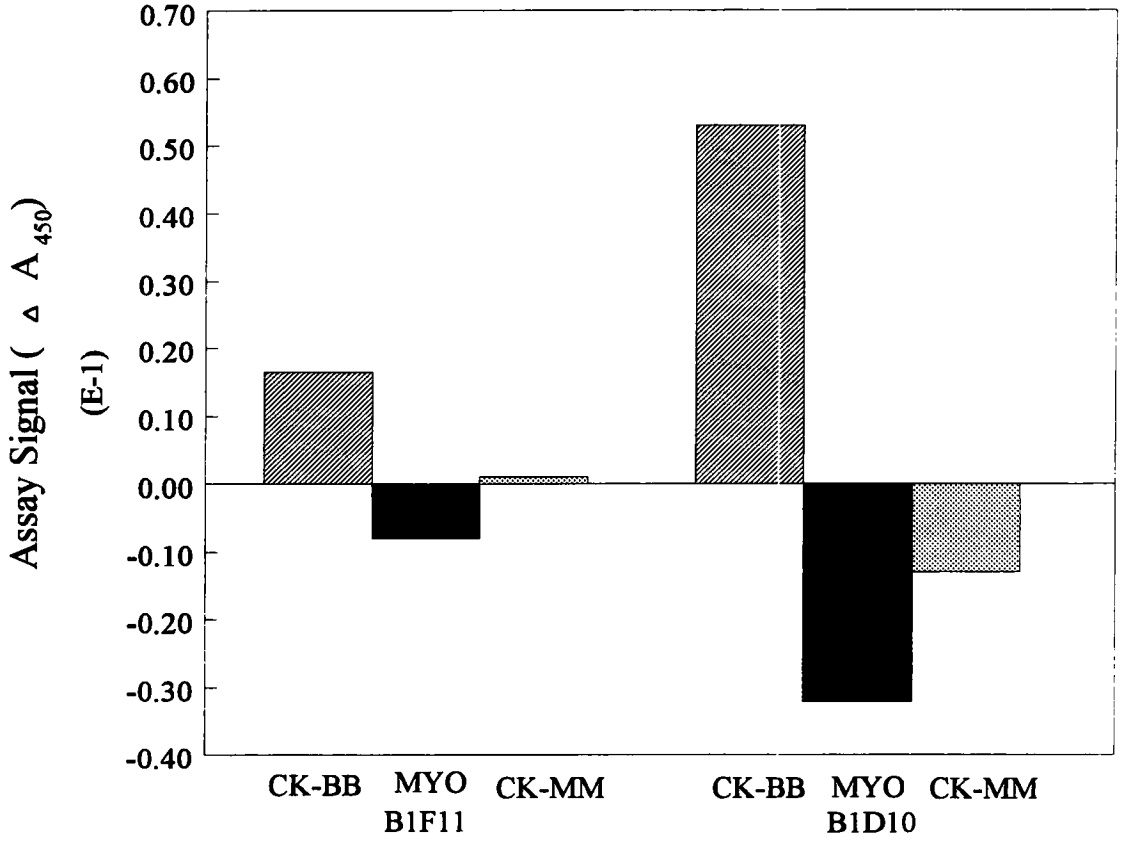


Fig. 14 Triple Check of CK-B Antibodies

Immulon II microwells were coated with 200 µL of 1 µg/mL CK-B antibodies, B1F11 and B1D10, were incubated for two more hours. Finally, 5,000-fold dilution of goat anti-mouse IgG-HRPO conjugate was added to provide enzyme activity. All of the data bars were represented by the mean of quadruplicate samples.

CK-MM
MYO
CK-BB

Table 3. Summary of Antibody Production *in vivo*

Clone	Days after abdomen I.P. of Hybridoma	Total of Ascites (mL)	Total Volume of Abdomen Washing (mL)	Total IgG (mg)	IgG Yield from Ascites Fluid (mg/mL)
B1D10	11	0.8	0	0.694	0.462
	13	0.7			
	15(death)	0			
B1D10	17	7	0	3.089	0.441
	19(death)	0			
B1F11	11	0.5	3	1.904	0.476
	12	0.5			
	17(death)				
B1F11	11	1	0	0.913	0.304
	13	2			
	16(death)				
M1D6	20	1.5	0	2.222	0.694
	21	0.7			
	22	0.5			
	24	0.5			
	25(death)				
M1D6	12	1	3	2.475	0.619
	20(death)	Solid Tumor			
M1G9	7	0.5	0	0.516	0.344
	9	1			
	13(death)				
M1G9	9(death)	Solid Tumor	3	2.33	0.777
M1G9	10	0.1	0	1.85	0.638
	13	0.1			
	19	1.2			
	20	1.5			
	21(death)				
M2B7	11	3	0		
	12	1			
	14	0.2			
	15	0.2			
	16(death)				

1. Total amount of protein = (A_{280}/1.35) x final volume [65].
2. Average yield of IgG = total IgG/ total voulme

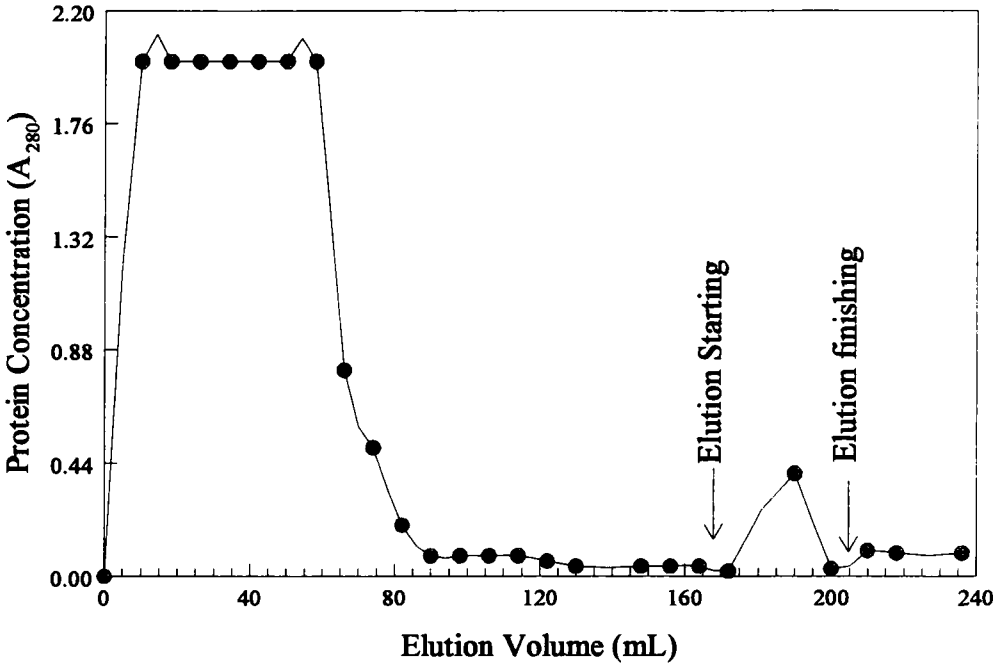


Fig. 15 Elution Profile of Purified Antibody from Protein A Affinity Chromatography

PBS was ultilized for column wash and citric phosphate pH 3.0 buffer was added to elute antibodies.

purified IgGs from culture media is specific anti-CK IgGs, but more than 90% of purified IgGs from ascites belong to the specific anti-CK IgGs.

6. Conjugation of CK-M Antibody with Horseradish Peroxidase

Periodate oxdized HRPO, 1.0 mL of 10.0 mg/mL, was conjugated to 1.0 mL of 2.0 mg/mL M1D6, 0.5 mL of 2.0 mg/mL M1G9, and 0.5 mL of 2.0 mg/mL M2B7, respectively, to yield 2 mL of M1D6-HRPO, 1 mL of M1G9-HRPO, and 1 mL of M2B7-HRPO. The optimal dilution studies of HRPO conjugated CK-M antibodies, M1D6-HRPO, M1G9-HRPO, and M2B7-HRPO, yielded 20,000, 5,000, and 4,000 folds dilution, respectively (data not shown). These data were then used in the preliminary study of ELISA.

C. ELISA Condition Study

1. Preliminary Study

(A) Saturation study

CK-B antibody, B1D10, was diluted serially, and then 200 μL of the diluted antibody solutions were incubated in Immulon I microwells at room temperature for 16 hours. After the assay, the saturating concentration of B1D10 on Immulon I microwells was determined to be 1.25 mg/mL (Fig.16).

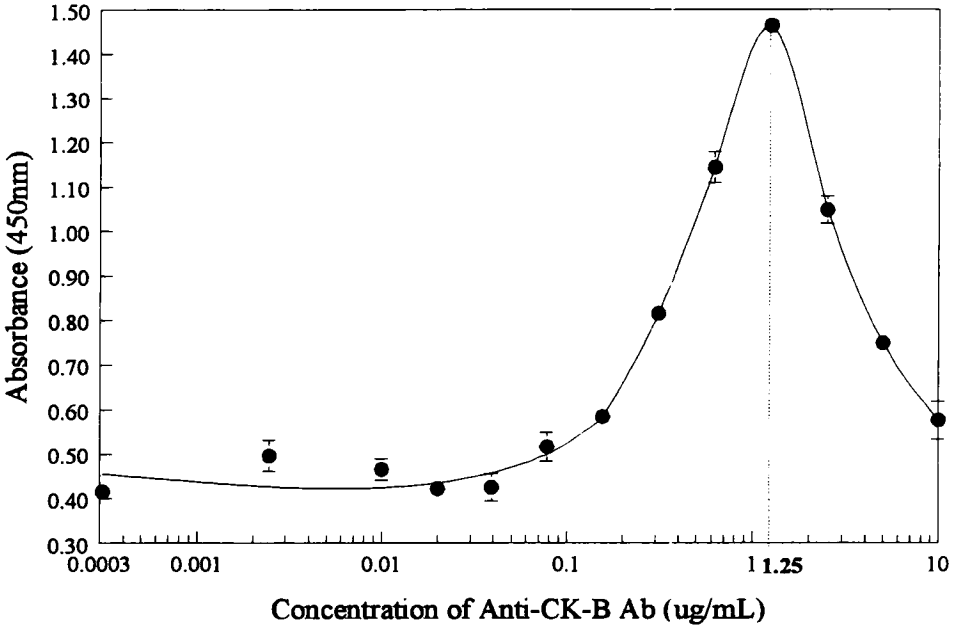


Fig. 16 Saturation Study of the Primary Antibody on Microwells
CK-B antibody, B1D10, was prepared from 0.0003 to 10 μg/mL in coating buffer and these solutions were incubated in Immulon I microwells at room temperature for 16 hours. Goat anti-mouse IgG-HRPO conjugate was prepared in 5,000-fold dilution for 1 hour.

(B) Coating time study

On Immulon I microwells, 200 μL of 1.25 μg/mL of B1D10 antibody was incubated for 2, 4, 6, 7, 14, 16, 18, and 20 hours. The optimal coating time for Immulon I microwell was determined to be 16 hours (Fig. 17A).

(C) Pairing study

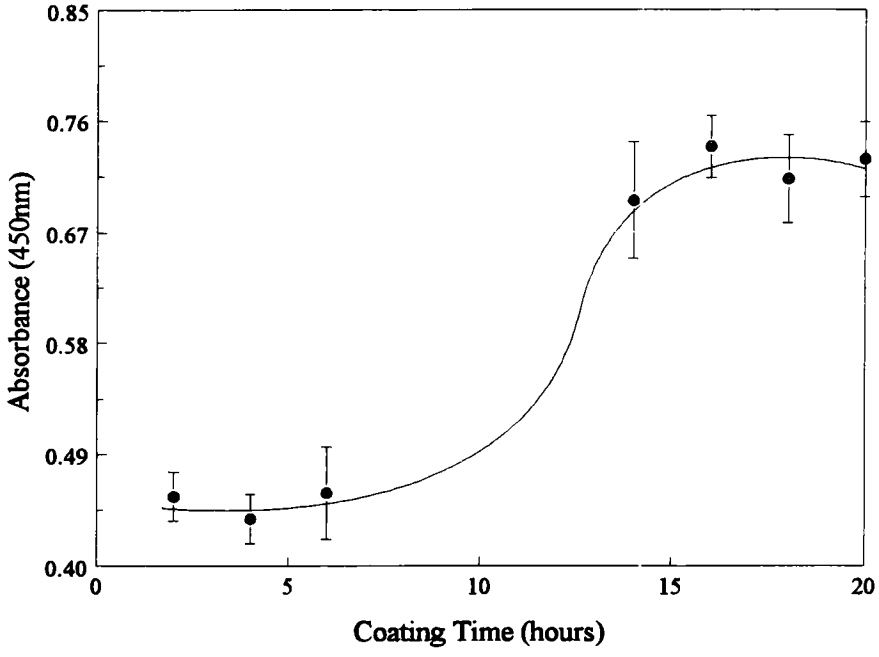
Because the antibody pairing of sandwich assay is relative to the antibody-epitope binding orientation, the pairing study was necessary for determining the optimal pairing for the CK-MB ELISA system. From the triple screening studies, B1D10 antibody was concluded to be a CK-B antibody with good specificity. The pairing of B1D10 antibody with three CK-M antibodies were studied to determine the best pairing. In the paring study, 200 μL of 1.25 μg/mL B1D10 antibody was incubated on Immulon I microwells for 16 hours.

After incubation of CK-MB, 20,000, 5,000, and 4,000 folds of diluted M1D6-HRPO, M1G9-HRPO, and M2B7-HRPO solutions were incubated in each of the CK-MB bound microwells for another hour. As the data indicates (Fig. 18), M1D6 shows the best pairing specificity with B1D10, thus, B1D10–CK-MB–M1D6 was selected for the proposed ELISA system.

(D) Blocking agent study

Different concentrations, 0.5%, 1%, and 3%, of BSA, OVA, pig albumin, horse albumin, and skim milk solutions were used to study their 2 hour-blocking effect on Immulon I microwells. The results showed that 0.3% OVA was the most effective

A.



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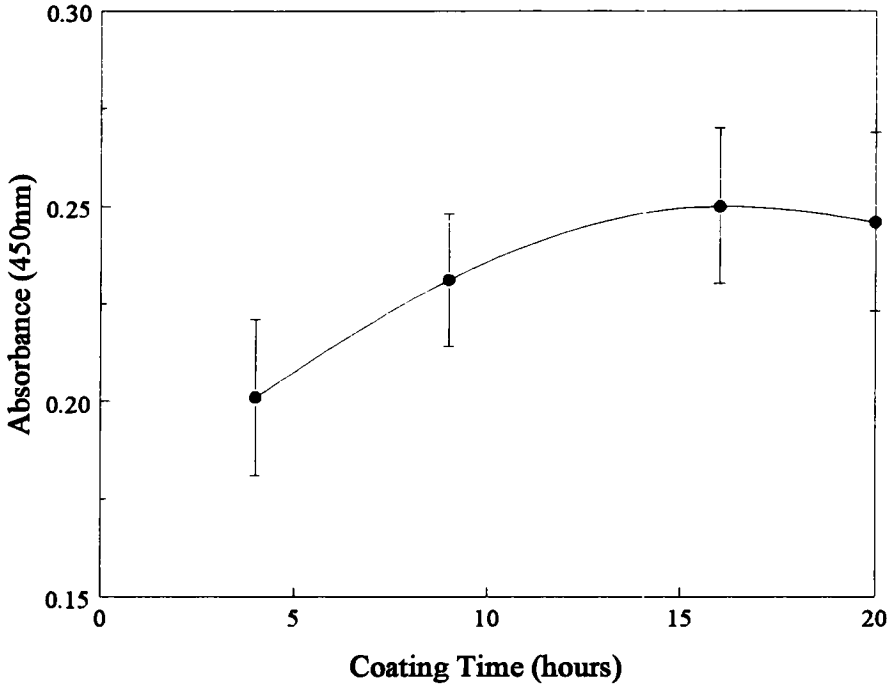


Fig. 17 Optimal Coating Time of CK-B Antibody

A. CK-B antibody, B1D10, was coated on Immulon I microwells by 200 μL/well of 1.25 μg/mL for 2-20 hours. **B.** B1D10 was prepared in the concentration of 0.5 μg/mL and coated on Immulon II microwells for 4-20 hours.The optimal coating time for these two microwells both are proximately 16 hours.

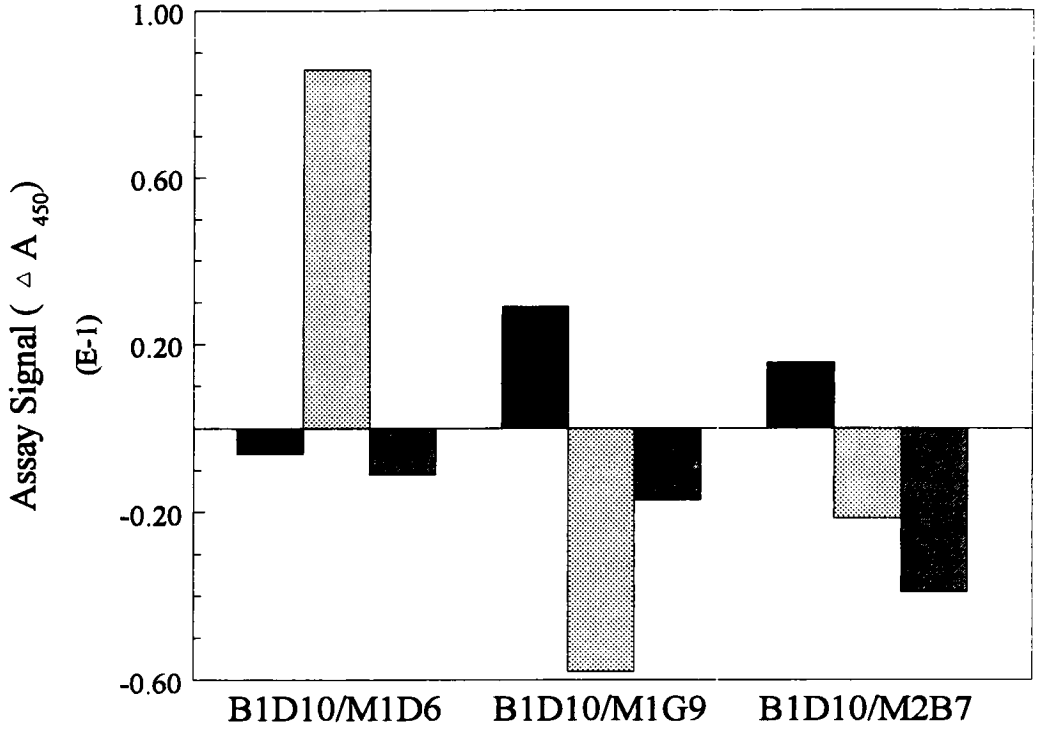


Fig. 18 Pairing Study of ELISA System
On B1D10 coated and BSA blocked Immunolon I Microwells, HRPO conjugated CK-M antibodies, M1D6-HRPO, M1G9-HRPO, and M2B7-HRPO, were diluted in 20,000, 5,000, and 4,000 folds and incubated for 1 hour to complete the assay. All of the data were performed quadruplicately in a within-batch operation.

- CK-MM
- CK-MB
- CK-BB

blocking reagent in all of the studied concentrations; however, the background was so high that the signal to noise ratio decreased (Fig 19). Compared to OVA, higher signal to noise ratio was found with 0.3% BSA because of its lower background. Hence, from these results, BSA was chosen to be the blocking agent for Immulon I microwells.

(E) Washing agent study

Deionized water, PBS, PBS + 0.05% Tween 20, and PBS + 0.1% Tween 20 were considered as washing agents for the ELISA system. Each washing agent followed by deionized water, were applied for 3, 5, and 7 times respectively. The results indicated that the optimal washing agent and condition was 3 times with PBS washing followed by 3 times with deionized water (Fig. 20).

2. Solid Phase Study

Five types of removable microwell strips, Immulon I, II, and IV, Nunc, and Corning, were evaluated for the optimal solid phase for the proposed ELISA system.

(A) Saturation study

To each of the microwells, 200 μL of varying concentrations (0.01-10 μg/mL) of B1D10 antibody solutions were coated at room temperature for 16 hours. From the results of the saturation study, the optimal coating concentrations for Immulon I, II, and IV, Nunc, as well as Corning microwells were 1.25, 0.5, 1, 1.25, and 1.25 μg/mL, respectively (Table 4).

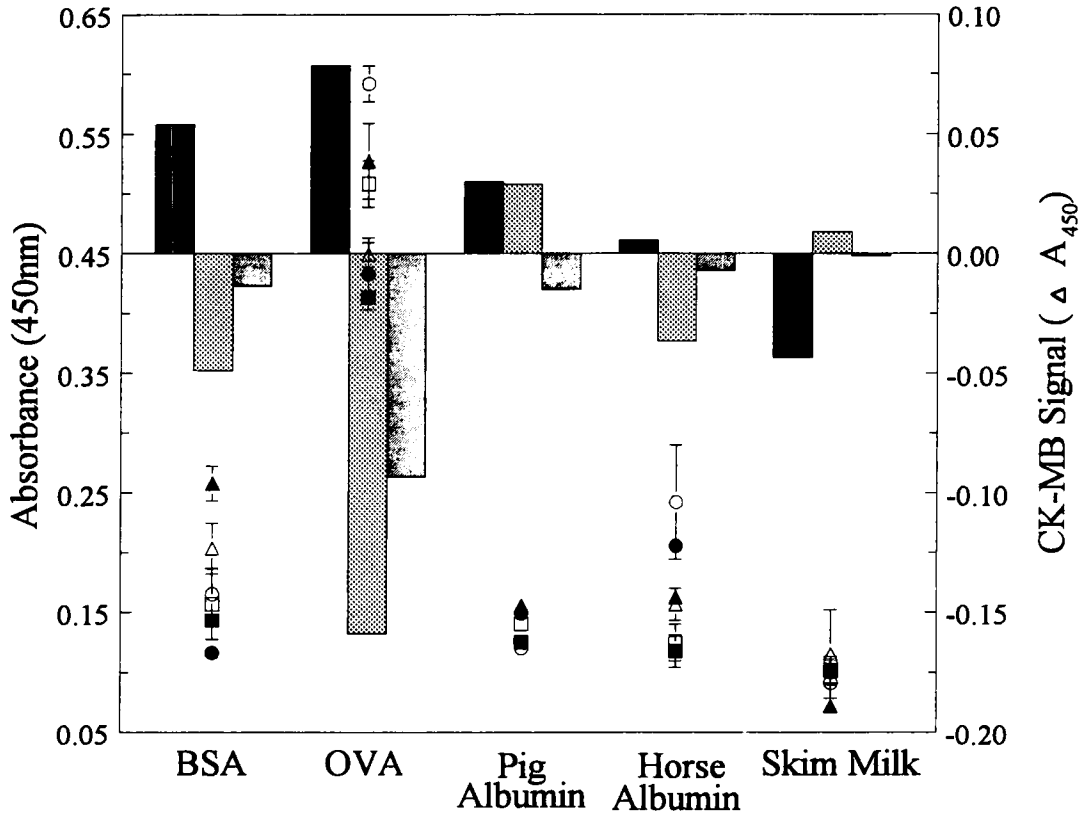


Fig. 19 Blocking Agent Study

BSA, OVA, pig albumin, horse albumin, and skim milk were prepared in 0.5%, 1%, and 3%, and 200 μL of these blocking solutions were incubated in B1D10 coated Immulon I for 2 hours. Left Y axis showed the sample reading and blank reading at 450 nm. Right Y axis showed the assay signal from the difference between sample and block

CK-MB Signal of 0.5, 1, 3% Blocking

Sample Reading of 0.5, 1, 3% Blocking

Blank reading of 0.5, 1, 3% Blocking

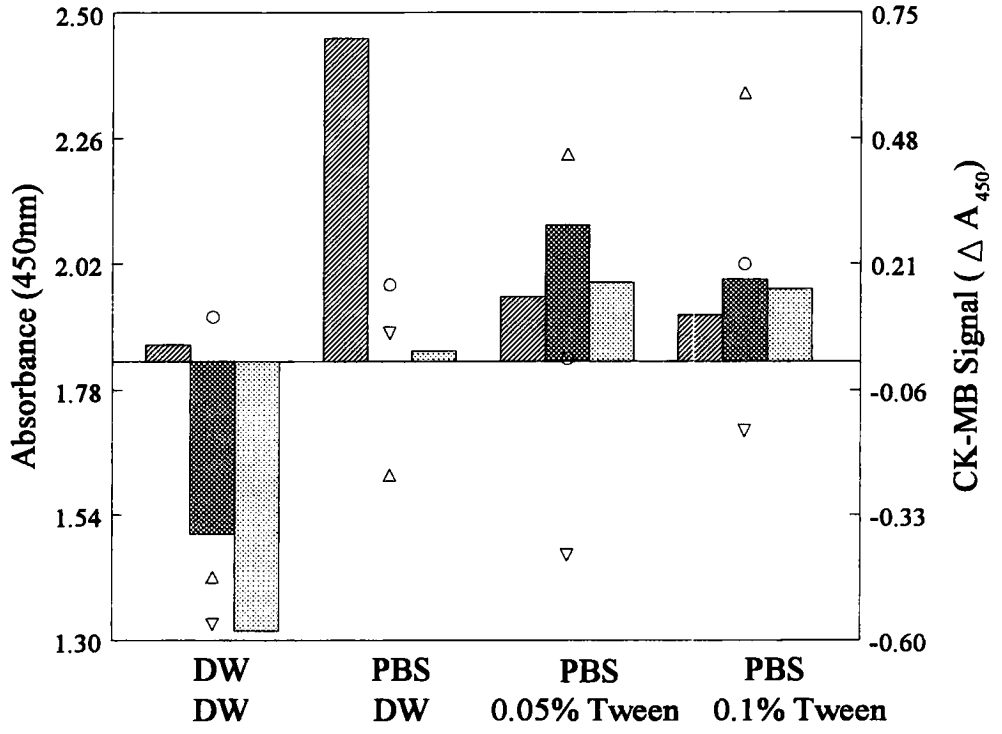


Fig. 20 Washing Agent Study

Different washing combinations were applied for optimal wash. Left axis showed the blank reading at 450 nm; right Y axis was represented the CK-MB signal from the difference between sample reading and blank reading.

CK-MB Signal of 3/3, 5/5, anf 7/7 Washing

Blank of 3/3, 5/5, and 7/7 Washing

Table 4 Summary of Saturating Concentrations of CK-B Antibody B1D10

Microwells	Saturating Concentration of B1D10 (μg/mL)[1]
Immulon I	1.25
Immulon II	0.50
Immulon IV	1.00
Nunc	1.25
Corning	1.25

1. The whole process was proceeded at room temperature (20°C) for 16 hours.

(B) Coating time study

On Immulon I and II microwells, 200 μL/well of 1 and 0.5 μg/mL B1D10 antibody solutions were coated respectively for different times. The optimal coating time for both was determined to be 16 hours (Fig. 17).

(C) Microwell performance study

After the prior blocking agent study in Immulon I microwells, BSA and OVA were found to be effective blocking agents for the ELISA system. The further blocking study for different types of microwells under different conditions was investigated. The results indicated that effective blocking was obtained at 3 hours incubation of 3% BSA for Immulon I microwell, 4 hours incubation of 0.5% OVA for Immulon I microwell, 4 hours incubation of 0.5% BSA for Immulon II microwell, 4 hours incubation of 1% OVA for Corning microwell, and 1 hour incubation of 3% OVA for Immulon IV microwell (Table 5). After repeating these assays several times, 3 hours incubation of 3% BSA on Immulon I microwells was shown to give the optimal ELISA performance (Fig. 21).

(D) Blocking effect study of different types of BSA

High background and low CK-MB signal were the main problems in this ELISA system. As indicated above for Immulon I microwells, the 3 hours incubation of 3% BSA was found to be the most effective blocking conditions. Since several types of BSA were available from different preparations, a study was performed to determine the type of BSA that can provide the best blocking effect. Five types of

Table 5 Summary of Well Performance and Blocking Study

Well Type	Optimal Blocking Agent	Blocking Time (hours)	Assay Noise $N = (A_{450})_0$	Assay Value $S = A_{450} - (A_{450})_0$	S/N Ratio
Immulon I (1.25 μg/mL)	3% BSA	4	1.077±0.106	0.007±0.139	0.007
		3	0.998±0.019	0.231±0.039	0.231
		2	1.022±0.098	0.083±0.119	0.081
		1	1.192±0.020	-0.252±0.084	–
	0.5% OVA	4	1.449±0.012	0.209±0.070	0.144
		3	1.691±0.057	-0.016±0.060	–
		2	1.650±0.038	0.152±0.042	0.092
		1	1.786±0.001	0.004±0.038	0.002
Immulon II (0.5 μg/mL)	0.5% BSA	4	0.262±0.034	0.259±0.024	0.989
		3	0.046±0.012	0.055±0.014	1.196
		2	0.418±0.054	0.096±0.055	0.230
		1	0.443±0.022	0.038±0.028	0.086
Corning (1.25 μg/mL)	1% OVA	4	0.926±0.010	0.294±0.022	0.317
		3	0.074±0.026	0.101±0.050	1.365
		2	0.966±0.024	0.094±0.027	0.094
		1	1.177±0.013	0.037±0.014	0.031
Immulon IV (1 μg/mL)	3% OVA	4	0.591±0.054	-0.212±0.058	–
		3	0.504±0.003	0.068±0.019	0.134
		2	0.444±0.038	0.116±0.038	0.261
		1	0.536±0.030	0.194±0.041	0.362
Nunc (1.25 μg/mL)	3% OVA	4	0.334±0.037	-0.266±0.049	–
		3	0.458±0.026	0.065±0.061	0.142
		2	0.580±0.005	-0.041±0.092	–
		1	0.457±.0.029	0.022±0.029	0.048

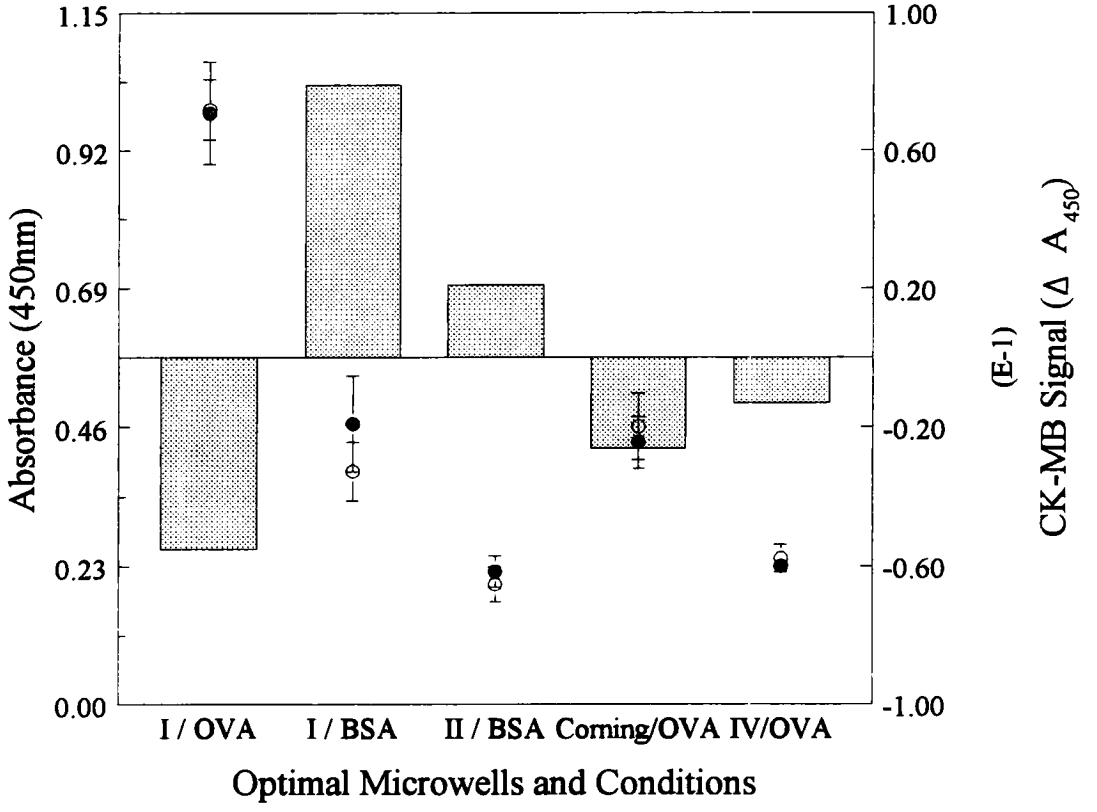


Fig. 21 Optimal Microwell Performance Study

Solid phases, Immulon I, II, and IV, as well as Corning, were coated by 1.25, 0.5, 1.0, 1.25 μg/mL of B1D10 for 16 hours. Immulon I microwells were blocked by 3% BSA for 3 hours and by 0.5% OVA for 4 hours. Immulon II microwells were blocked by 0.5% BSA for 5 4 hours. Immulon IV microwells were blocked by 1% OVA for 4 hours. The absorbances at 450 nm of sample and blank were showed by left Y axis and the CK-MB signals were represented by right Y axis from the difference between sample and blank reading.

CK-MB Signal

Blank

Sample

BSA, A-2153, A-3059, A-4503, A-7030, and A-7906, from Sigma Chemicals were subjected to this study. The results of this study indicated that 3% BSA of A-3059 incubated for 3 hours can provide the best blocking effect (Fig. 22).

(E) Blocking condition study

The optimal blocking condition of BSA (A-3059) was checked again at the concentrations of 0.5%, 1%, 3%, and 5% on Immulon I microwells for 1.25, 2, 3, and 4 hours incubations. From the signal to noise ratio (S/N), 1.25 hours incubation of 3% BSA (A-3059) were the optimal blocking conditions (Table 6).

3. Antigen Study

(A) Optimal incubation buffer study

Because CK-MB isoenzyme is unstable, it is very important to use a buffer that will stabilize the CK-MB incubation. Different buffers, PBS, 2 mM 2-mercaptoethanol in PBS, 0.1% BSA in PBS, 0.5% BSA in PBS, 1% BSA in PBS, 1 mM reduced glutathione in PBS, 0.1% BSA + 1 mM reduced glutathione in PBS, 0.5% BSA + 1 mM reduced glutathione in PBS, and 1% BSA + 1 mM reduced glutathione in PBS, were studied. The results indicated that the buffer containing 0.1% BSA + 1 mM reduced Glutathione in PBS was the optimal stabilizing buffer system for the antigen incubation (Fig. 23).

(B) Optimal incubation time study

In each Immulon I microwell which had been coated by 300 μL of 1.25 μg/mL B1D10 antibody for 16 hours and then coated by 3% BSA A-3059 for 1.25 hours, 300

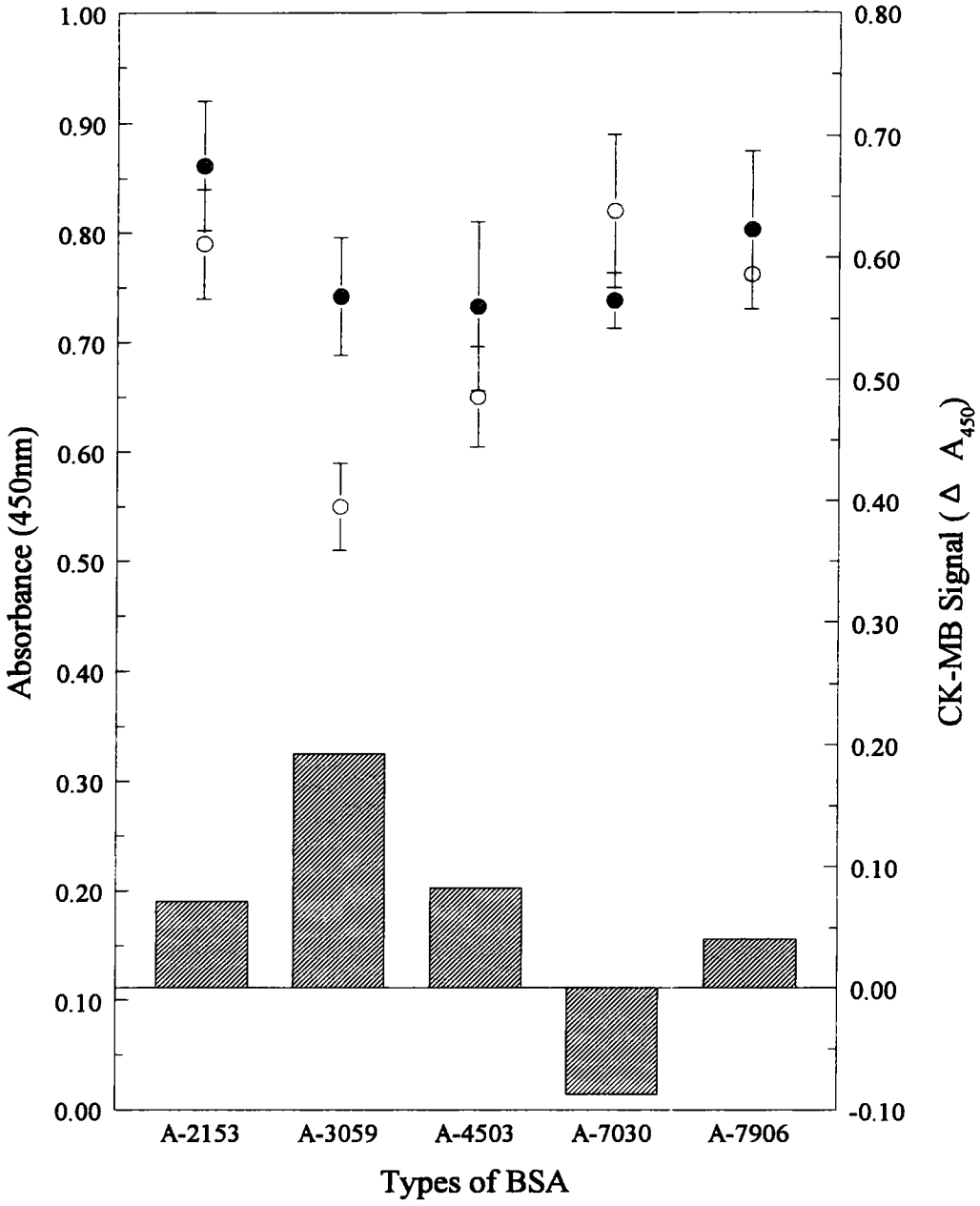


Fig. 22 Blocking Study of Different Types of BSA
Different types of BSA were utilized by the blocking condition: 3% and 3 hours. The sample reading and blank reading were showed by left Y axis and CK-MB was represented by right Y axis.

▨ CK-MB Signal

○ Blank

● Sample

Table 6 Summary of Blocking Study of Different Types of BSA

BSA Types	Optimal Concentration (%)	Blocking Time (hours)	Assay $N = (A_{450})_0$	Assay Value $S = A_{450} - (A_{450})_0$	S/N Ratio
A-4503	**5**	2	0.907	0.058	0.064
	3	2	0.796	0.164	0.206
	1	2.5	1.162	0.094	0.081
		2	0.780	0.174	0.223
	0.5	3	0.821	0.073	0.089
		2	0.837	0.104	0.124
		1.25	0.763	0.126	0.165
A-3059	5	3	1.044	0.071	0.068
		1.25	0.803	0.145	0.181
	3	2.5	0.743	0.160	0.215
		2	0.952	0.126	0.132
		1.5	0.845	0.226	0.267
		1.25	**0.767**	**0.330**	**0.431**
	1	3.5	0.778	0.195	0.251
		2.5	0.817	0.200	0.245
		1.25	0.832	0.128	0.154
	0.5	2.5	0.747	0.193	0.258
		1.5	0.766	0.153	0.202

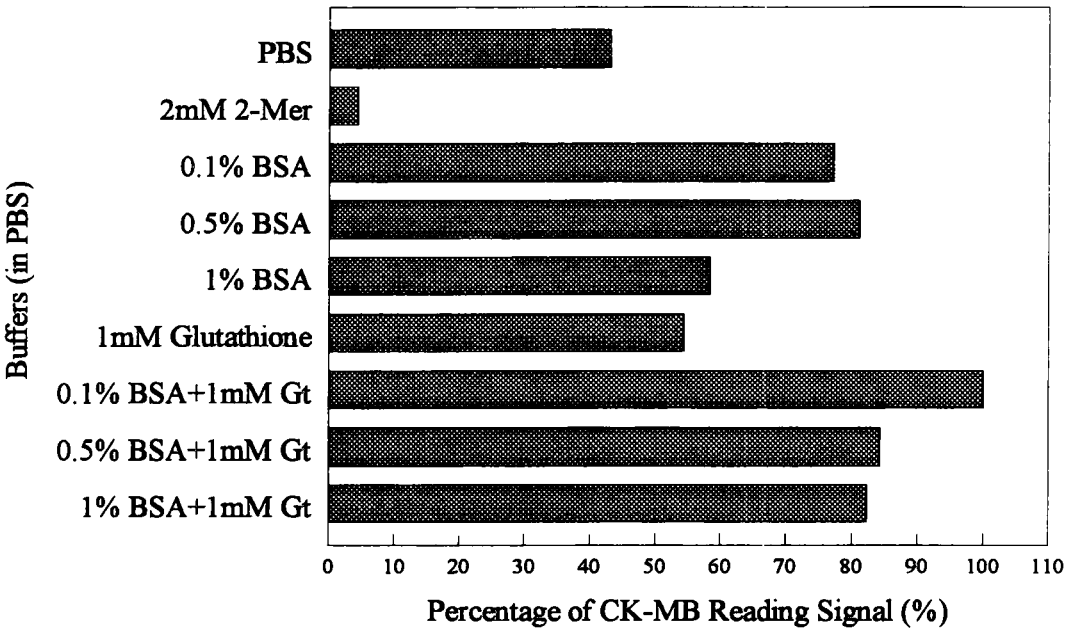


Fig. 23 Optimal CK-MB Incubation Buffer Study
All of the incubation buffer contained 100 ng/mL CK-MB and the CK-MB solutions were incubated for 1 hour. (Gt: Glutathione)

μL of different concentrations of CK-MB, ranging from 1.25 to 25 ng/mL, were added and incubated for 30, 60, and 90 minutes. The results of the assay indicated that the 60 minutes incubation of CK-MB provided a more stable, wider and more linear range for CK-MB ELISA system (Fig. 24).

4. HRPO Conjugated CK-M Antibody Study

(A) Optimal dilution study

In the CK-MB bound microwells, 200 μL of 500, 1,000, 2,000, 5,000, 10,000, 15,000, 20,000, 25,000, 30,000, and 40,000 folds diluted solutions of HRPO conjugated CK-M antibody, M1D6-HRPO, were added and incubated at room temperature for 60 minutes. The optimal CK-MB readings in the ELISA system was obtained at 5,000-fold dilution of M1D6-HRPO (Fig. 25).

(B) Optimal Incubation Buffer Study

To buffer the incubation of 5,000-fold dilution of M1D6-HRPO, 0%, 0.1%, 0.5%, 1%, and 2% BSA in PBS were used. The optimal buffer effect for 5,000-fold dilution of M1D6-HRPO was provided by 0.1% BSA in PBS (Fig.26).

(C) Optimal incubation time study

In a series of 0.63, 1.25, 2.5, 5.0, 10, 15, and 20 ng/mL of CK-MB bound Immulon I microwells, 300 μL of 5,000-fold diluted M1D6-HRPO solution was added and incubated for 30, 60, and 90 minutes. In this study, the assay was performed under

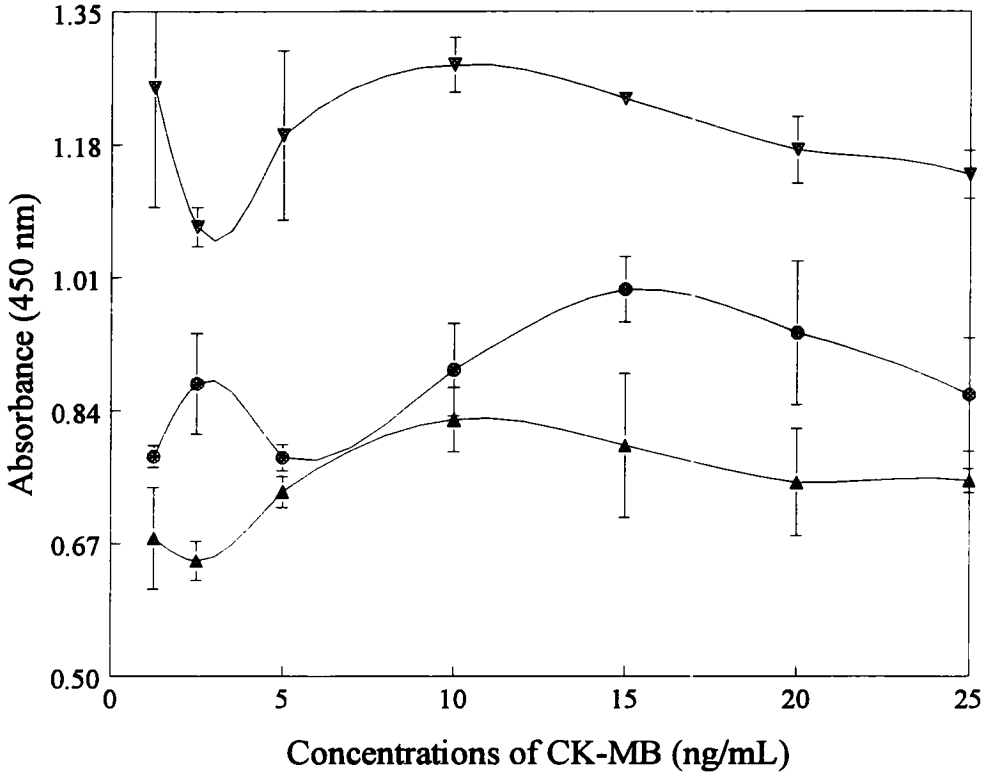


Fig. 24 Optimal CK-MB Incubation Time Study
CK-MB solution within linear range, 1.25-20.0 ng/mL, were prepared in 1 mM reduced glutathione + 0.1% BSA in PBS for 30, 60, and 90 minutes.

▲ 30 minutes, Y = 0.602 + 0.023X, r = 0.984

● 60 minutes, Y = 0.676 + 0.021X, r = 0.9997

▼ 90 minutes, Y = 1.030 + 0.026X, r = 0.965

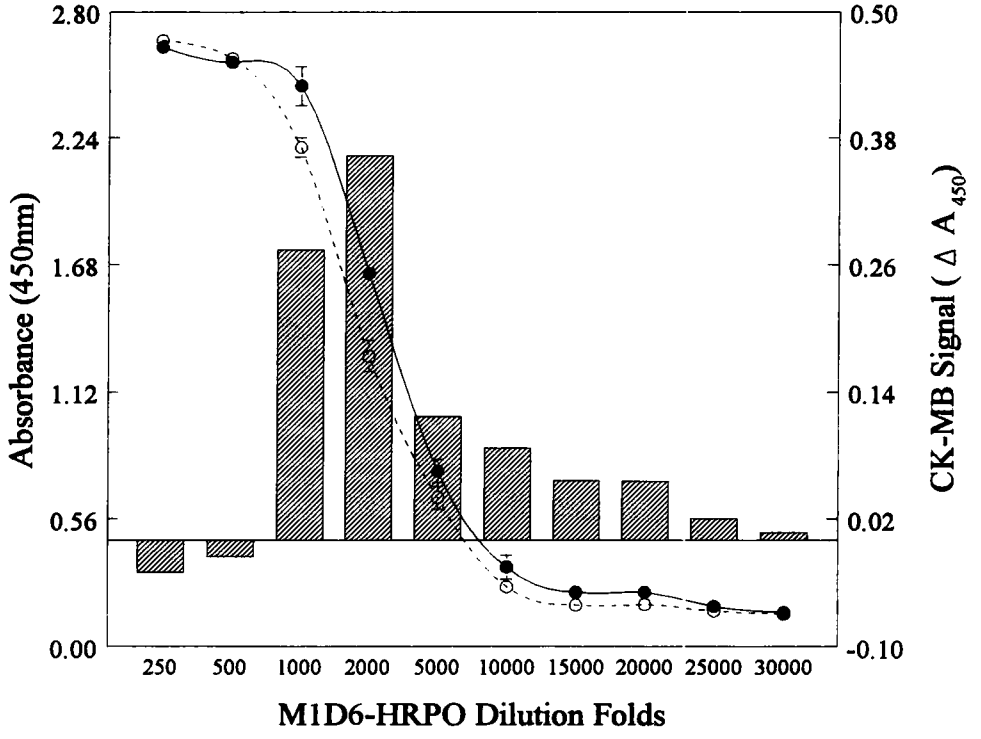


Fig. 25 Optimal Dilution Fold Study of HRPO Conjugated CK-M Antibody
M1D6-HRPO was diluted serially from 250 to 30,000 folds and incubated in CK-MB–B1D10 complex immobilized Immulon I microwells for 1 hour. The absorbance at 450 nm of sample and blank were showed by left Y axis and the CK-MB signal was represented by right Y axis by the absorbance difference between sample and blank.

▨ CK-MB Signal

○ Blank

● Sample

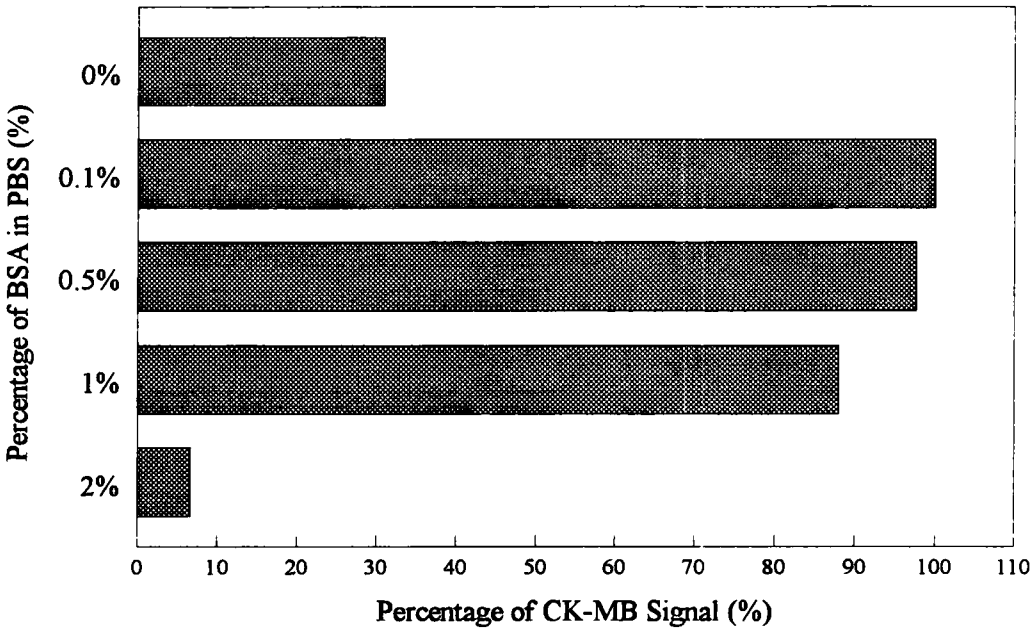


Fig. 26 Optimal Incubation Buffer Study of HRPO Conjugated CK-M Antibody M1D6-HRPO was prepared to be 5,000-fold dilution in different buffers and incubated for 1 hour. The absorbances at 450 nm of CK-MB samples and blanks and the CK-MB signals from the differences between samples and blanks were showed by left and right Y axises, respectively.

the optimized conditions. A good linear range of CK-MB was obtained after 90 minutes incubation (Fig. 27).

5. Post Incubation Washing Study

Because buffers were applied in the CK-MB and M1D6-HRPO incubations to stabilize these two enzymes, washing conditions needed to be adjusted in order to obtain the optimal ELISA signal. From the washing study, seven times with PBS wash followed by seven times with deionized water wash provided the optimal washing condition after the incubation of CK-MB in the PBS containing 0.1% BSA + 1 mM reduced glutathione. Similarly, 10 times with PBS wash followed by 10 times with deionized water wash provided the optimal washing condition after the incubation of M1D6-HRPO in 0.1% BSA contained PBS (Fig. 28A).

After the above adjustments, the washing conditions, 3, 5, and 7 times of PBS followed by the equal times of deionized water washing, respectively, were applied in washing for the well coating and blocking in the ELISA system. The results of this study indicated that 5 times with PBS and 5 times with deionized water washing, respectively, produced the optimal conditions (Fig.28B).

6. Color Development Time Study

The whole assay procedure was conducted under the optimal conditions. The final step of color development was stopped at the 1.5, 3, 5, 10, and 15 minutes after

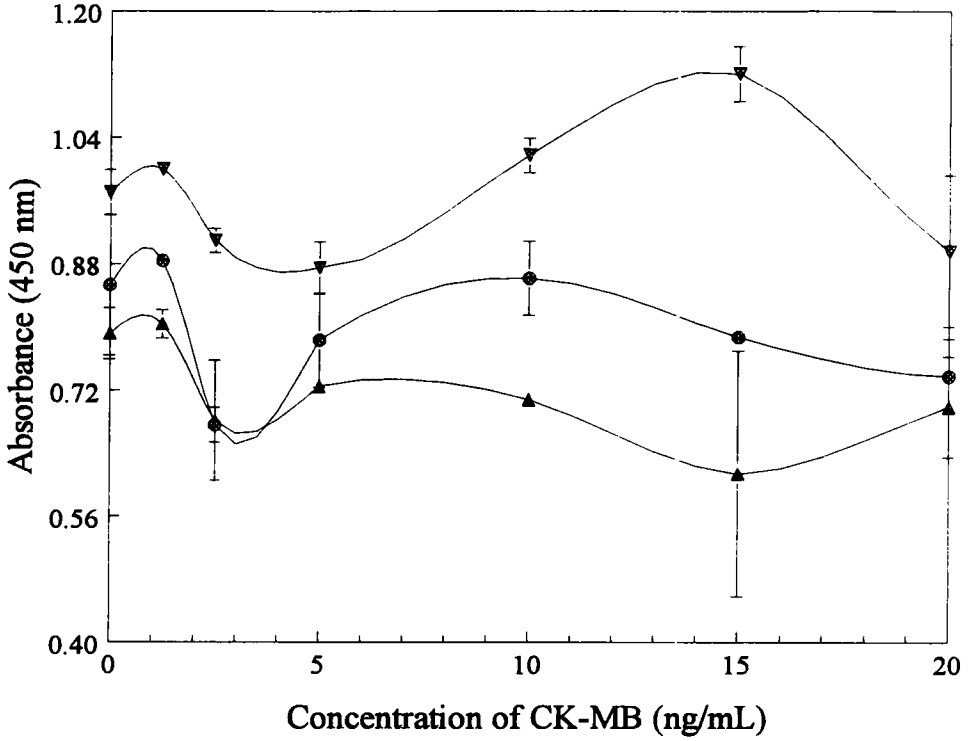


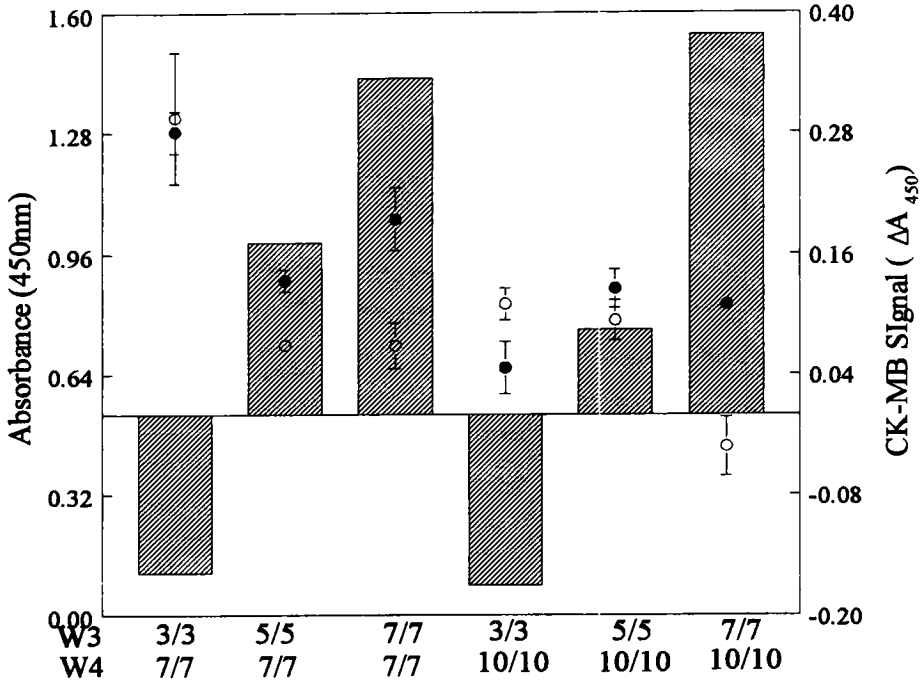
Fig. 27 Optimal Incubation Time Study of HRPO Conjugated CK-M Antibody CK-MB solution within linear range, 1.25-20.0 ng/mL, were bound in B1D10 immobilized Immulon I microwells, and then 5,000-fold dilution of M1D6-HRPO was incubated in 0.1% BSA contained PBS buffer for 30, 60, 90 minutes.

▲ 30 minutes

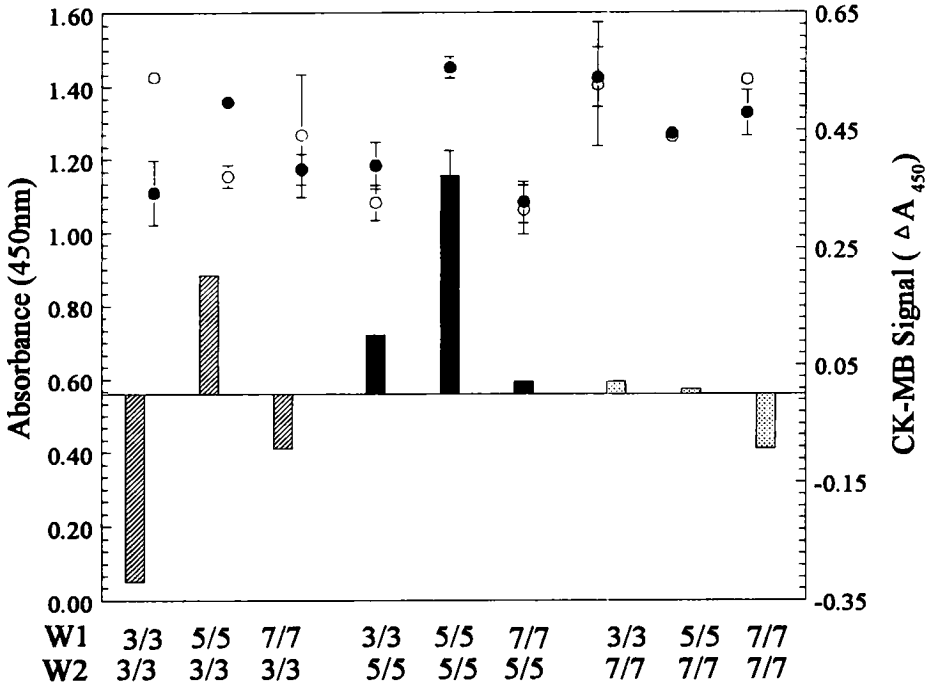
● 60 minutes

▼ 90 minutes, Y = 0.759 + 0.025X, r = 0.996

A.


1.60
1.28
0.96
0.64
0.32
0.00
Absorbance (450nm)
0.40
0.28
0.16
0.04
-0.08
-0.20
CK-MB SIgnal (ΔA 450)
W3 3/3 5/5 7/7 3/3 5/5 7/7
W4 7/7 7/7 7/7 10/10 10/10 10/10

B.


1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Absorbance (450nm)
0.65
0.45
0.25
0.05
-0.15
-0.35
CK-MB Signal (Δ A 450)
W1 3/3 5/5 7/7 3/3 5/5 7/7 3/3 5/5 7/7
W2 3/3 3/3 3/3 5/5 5/5 5/5 7/7 7/7 7/7

the mixing of solution A and solution B (substrate and chromagen solutions). The optimal CK-MB detection was obtained after 5 minutes (Fig. 29).

7. Summary of Optimal CK-MB Detecting ELISA System

After the series of ELISA condition studies were performed, the optimized ELISA system was established (Fig. 30). This system estimates the existing amount of CK-MB in the sample by antibody-recognized conformation, but not CK-MB enzyme activity.

D. Performance Characteristic Study of CK-MB Detecting ELISA System

1. Linearity Study

The linear CK-MB detection range was determined to be between 2.5 and 15 ng/mL (Fig. 31). When the concentration was over 15 ng/mL, the absorbance at 450nm decreased. At concentrations lower than 2.5 ng/mL, the absorbance fluctuated due to the nonspecific binding in the microwell.

2. Detection Limit Study

From this study, the high background readings induced by nonspecific binding in the ELISA assay narrowed the CK-MB detecting range even more. The results of the study were subjected to statistical analysis according to the recommendation of the International Federation of Clinical Chemistry [19], and the detecting limit was

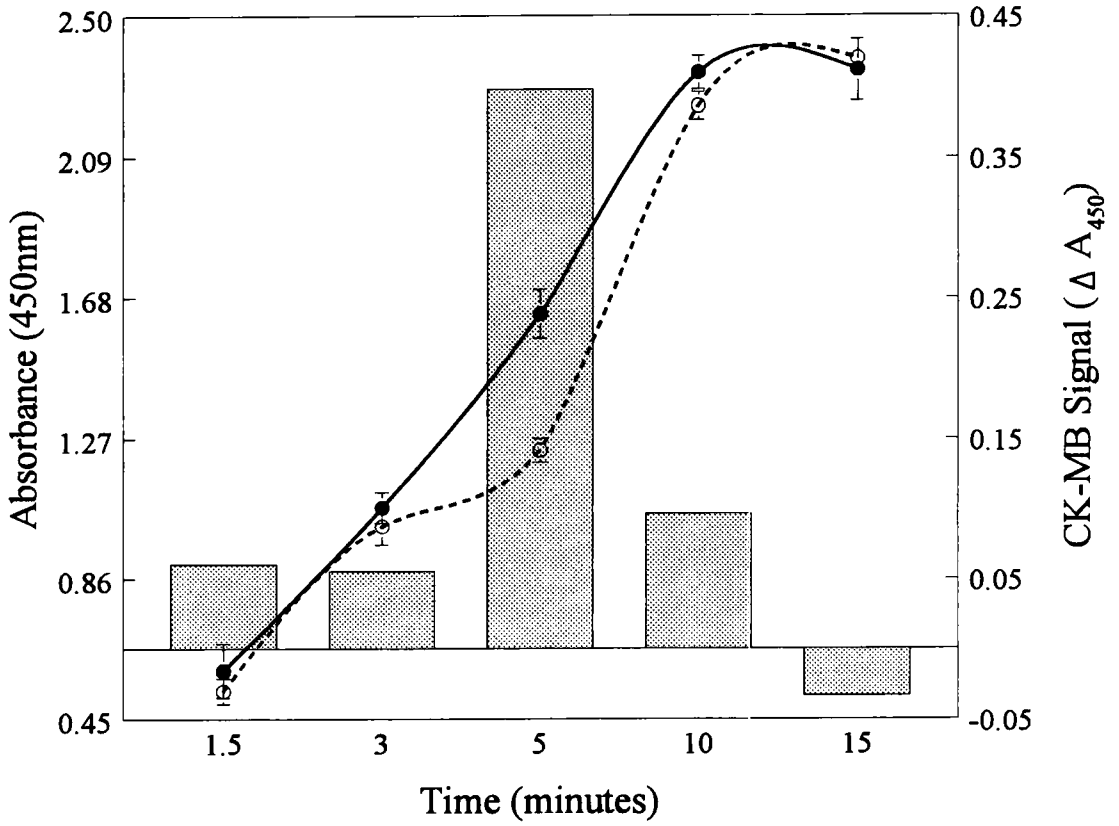


Fig. 29 Color Development Study

The ELISA was carried out in the optimized conditions and stopped at 1.5-15 minutes after the adding of solution A and B. The absorbance at 450 nm of sample and blank as well as CK-MB signal were represented by left and right Y axises, respectively.

CK-MB Signal

○ Blank

● Sample

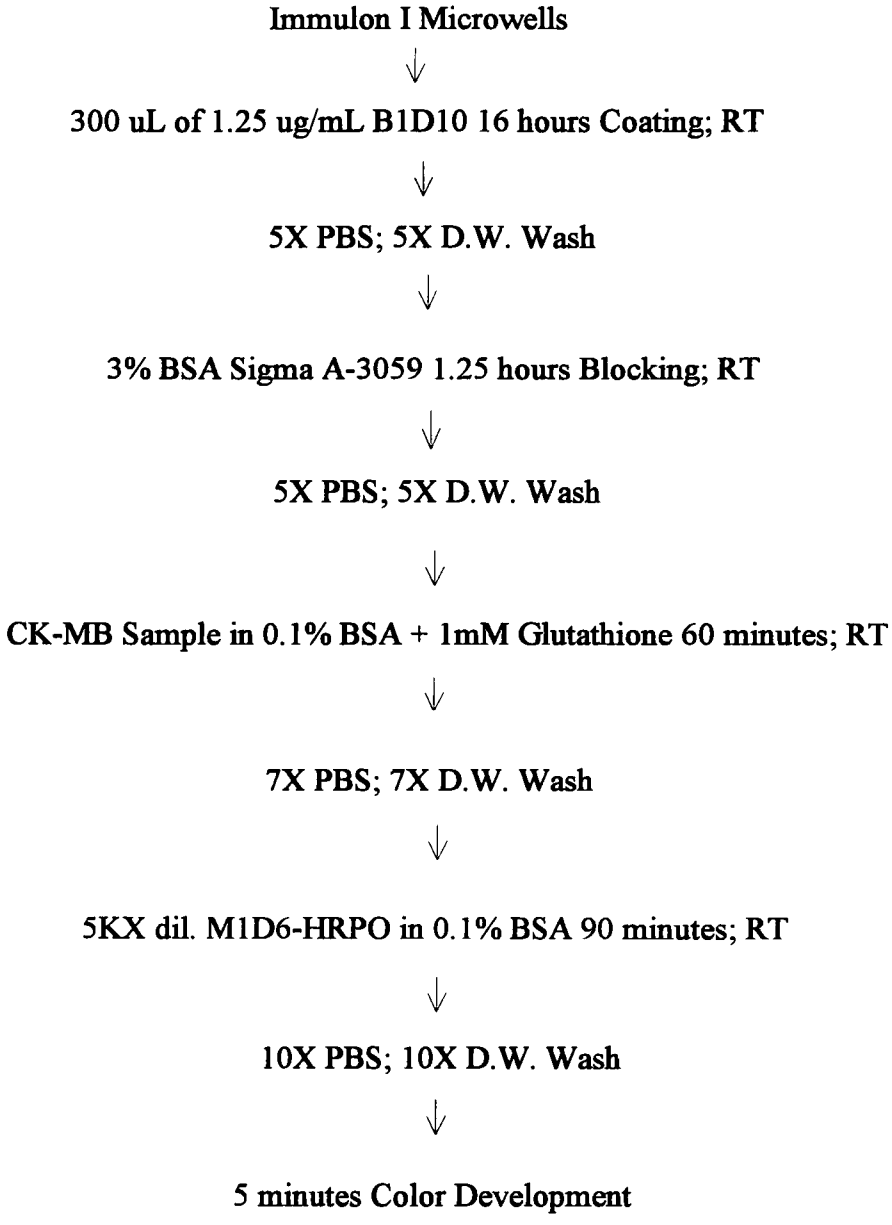


Fig. 30 Summary of CK-MB Detecting ELISA System

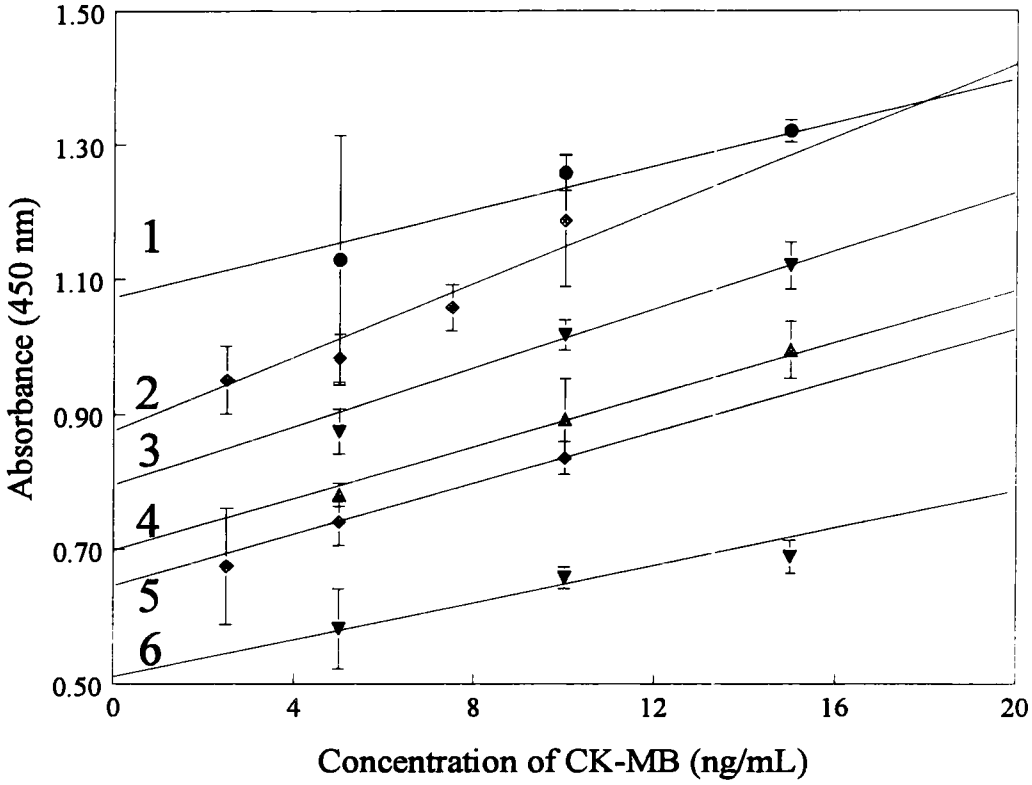


Fig. 31 Linearity Study of CK-MB Detection

From six independent within-batch operations of CK-MB linearity study, the CK-MB linear detection range was located between 2.5-15.0 ng/mL.

- ● Line 1: Y=0.019X + 1.07; r = 0.981
- ◆ Line 2: Y=0.031X + 0.87; r = 0.964
- ▼ Line 3: Y=0.025X + 0.799; r = 0.996
- ▲ Line 4: Y=0.021X + 0.676; r = 0.9996
- ◆ Line 5: Y=0.023X + 0.602; r = 0.984
- ▼ Line 6: Y=0.011X + 0.510; r = 0.972

determined to be 7.3 ng/mL while the blank reading (28 wells) at 450 nm was 0.896 ± 0.179 (standard deviation; SD = 0.069). The range of CK-MB detection was reduced to 7.3-15 ng/mL (Fig.32).

3. Precision Study

CK-MB, 300 μL of 10 ng/mL, was run in 24 replicated wells with a standard curve. The result of the precision study showed the ELISA system has a coefficient of variant (CV) of 12.3% at an CK-MB concentration of 10 ng/mL (Table 7).

4. Cross Reaction Study

The cross-reactions of CK-MM, CK-BB, myoglobin, and human serum albumin with the CK-MB ELISA system were studied. From the data obtained, there were no signal readings detected in the presence of CK-BB, myoglobin, and human serum albumin; however, an obvious signal reading appeared in CK-MM detection. The cross-reactivity of CK-MM with the CK-MB ELISA system was determned to be 4.8 % (Fig. 33).

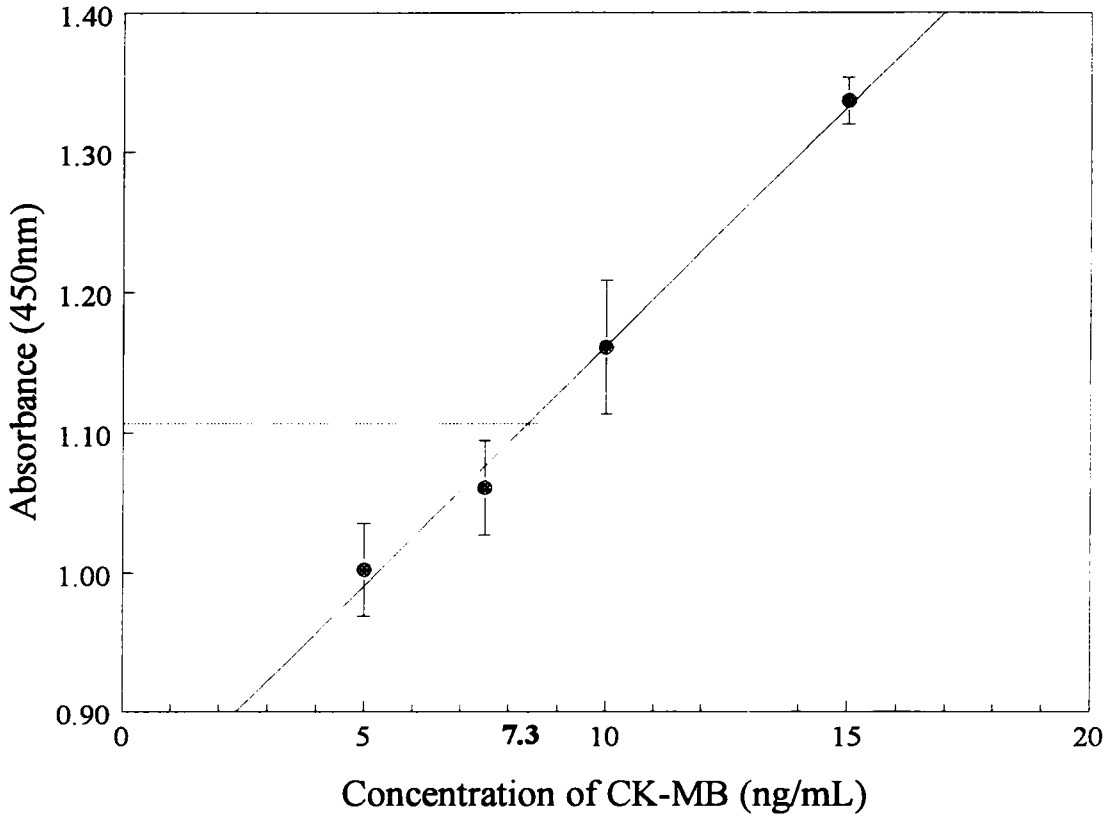


Fig. 32 Detection Limit Study

Twenty-eight microwells of 300 μL of CK-MB free incubation buffer were added into CK-BB antibody coated and BSA blocked Immulon I microwells. This incubation was carried out together with a linearity assay. From the readings of 28 blanks, the absorbance reading at 450 nm is 0.896 ± 0.1795. The detection limit of the ELISA is 7.3 ng/mL. The CK-MB detection linear range was decided to be 7.3-15 ng/mL and a linear standard curve was obtained, $Y = 0.815 + 0.034$, $r = 0.997$.

Table 7 Precision Study

Sample No.	24
	A_{450}
Mean	0.661
SD	0.081
Max.	0.779
Min.	0.529
C.V.	12.3%

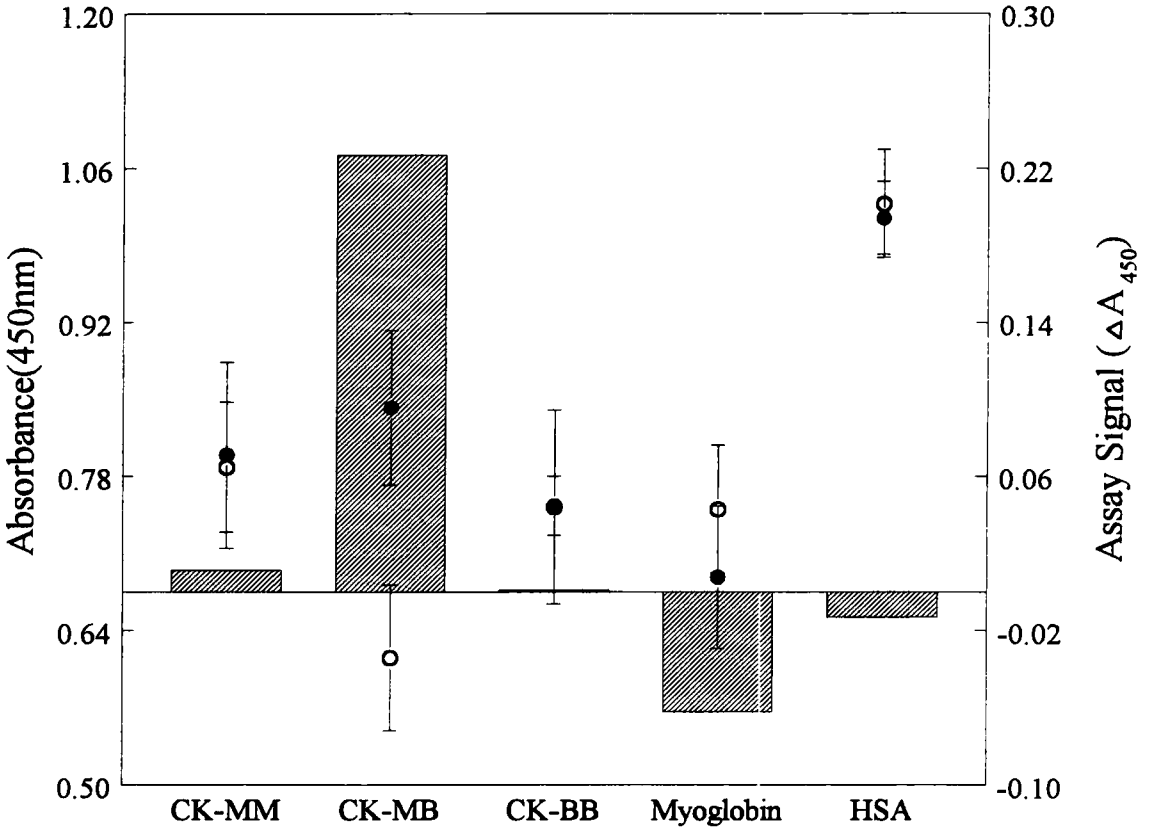


Fig. 33 Cross Reaction Study

CK-MM, CK-MB, CK-BB, myoglobin, and human serum albumin, 300 µL/well and 100 ng/mL, were incubated in B1D10 coated and 3% BSA blocked Immulon I microwells. The absorbances at 450 nm of samples and blanks, as well as assay signals from the differences between samples and blanks were showed by left and right Y axises, respectively. The cross-reactivity rate between CK-MM and the CK-MB detecting ELISA is 4.8%.

▨ Signal

○ Blank

● Sample

CHAPTER IV

DISCUSSION

A. Preparation of CK-MB Isoenzyme from Human Cardiac Tissue

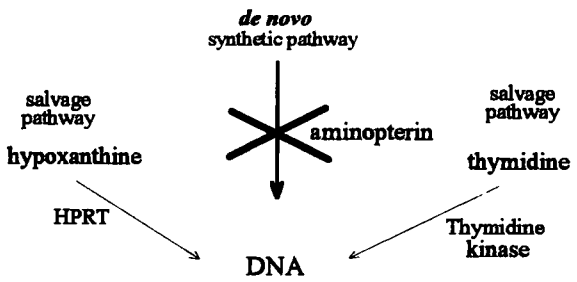
CK-MB isoenzyme was purified in our laboratory because of the high cost from commercial sources. DEAE-Sephadex A-50 ion exchange chromatography procedure was utilized for the isolation and purification of CK isoenzymes from human cardiac tissues [66, 67]. Extensive overlapping of CK-MM and CK-MB fractions were found in the first DEAE Sephadex ion exchange chromatography. The isolated CK-MB fraction was rechromatographed on the second and third DEAE-Sephadex ion exchange chromatographies with a lower linear gradient of NaCl (50-300 mM). About 50% of albumin contaminant that existed in the CK-MB isoenzyme fraction from the first ion exchange chromatography was removed by Affi-Gel Blue affinity chromatography [66, 67]. In spite of the high binding capacity with albumin, the nonspecific binding of Affi-Gel Blue resins will cause the loss of CK-MB isoenzyme in the process of affinity chromatography. Recently, an anti-human albumin affinity chromatography was reported to remove the albumin contaminant from CK-MB preparation more effectively [68].

B. Preparation of Monoclonal Antibodies

In the short term immunization of mouse, an intrasplenic antigen injection gave a direct and strong stimulation to induce the generation of antibody-secreting B cells in spleen within two weeks. A long term immunization, by prolonging the boost process and postponing the harvest of spleen cells, will induce a longer antibody plateau phase and thus more B cells with higher antibody-secreting titer can be obtained from mouse spleen. For the unstable CK isoenzyme, the antigen-antibody binding could decrease due to the change of the recognized antibody-binding epitope conformation on CK isoenzyme. Therefore, if the higher efficiency of generating monoclonal antibody-producing hybridoma clones can be improved, the higher possibility for finding the monoclonal antibody binding with CK on a stable binding epitope can be increased. The long term immunization seems a better strategy for this purpose, even though it could be time-consuming [53].

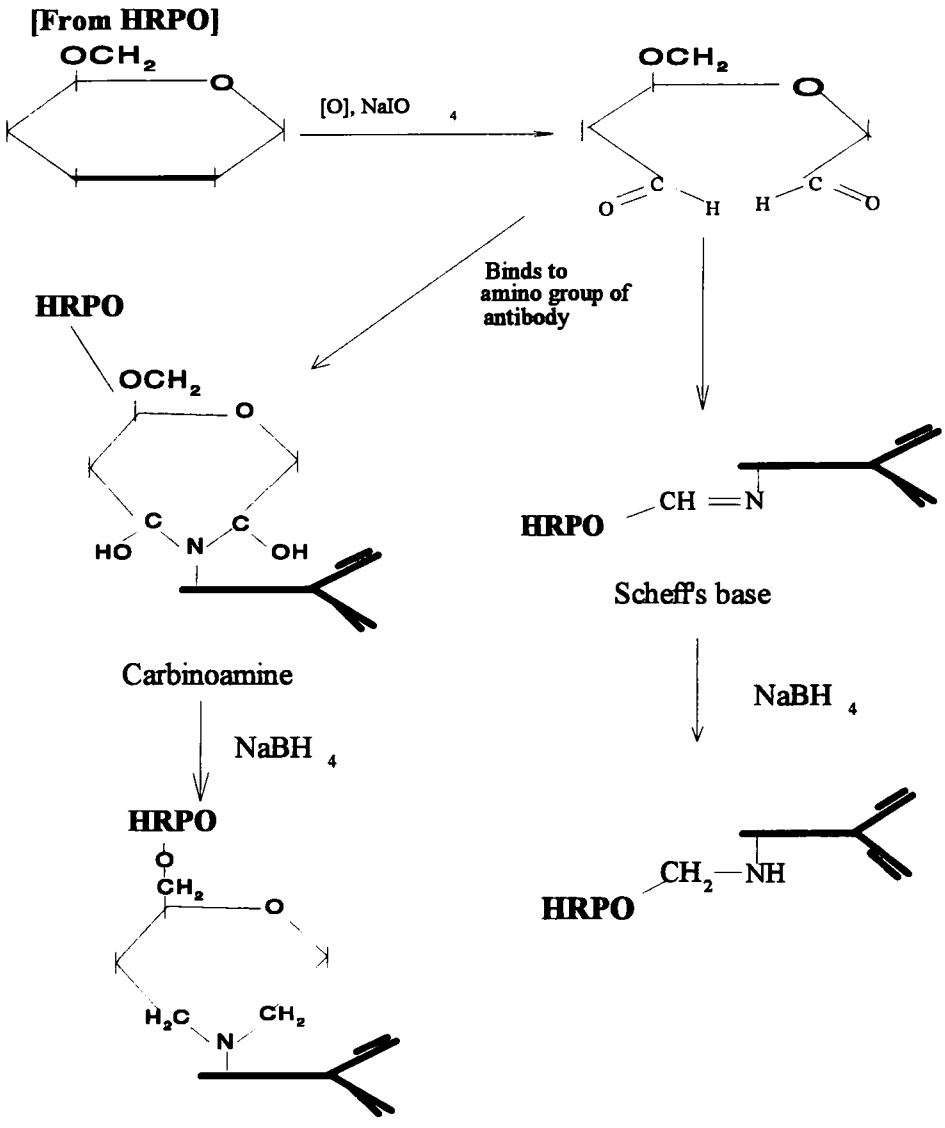
A BALB/c myeloma cell line, NS-1 cells, is a fast proliferating cell, but neither a secreting cell nor an intact antibody-producing cell (only a *kappa* light chain producer). When the hybridomas were fused from the immuned spleen cells and NS-1 myeloma cells, the antibody-producing characteristics of hybridoma cells derived from the immuned spleen cells, were combined with the fast proliferating capability from NS-1 cells. Generally, cell fusion for hybridoma generation is a random process. Three types of fused clones: NS-1 : NS-1, NS-1 : splenocyte, and splenocyte : splenocyte can be obtained in addition to the non-fused individual cells.

To select NS-1 : splenocyte hybridoma clones from the other fused clones, a HAT selection system was applied in the fused cell culture. HAT medium [53] which contains hypoxanthine, aminopterin, and thymidine was used in the culture medium for the purpose of selecting NS-1 : splenocyte hybridoma clones. Aminopterin, an analog of folic acid, inhibits the enzyme, dihydrofolate reductase, for *de novo* synthesis of purine and pyrimidine nucleotides as indicated in the following scheme. Thus, for hybridoma amplification, the salvage pathway becomes the only route for synthesizing purine and thymidine nucleotides for cell growth. The hybridoma cell, containing both hypoxanthine-guanine phosphoribosyl transferase (HPRT) and thymidine kinase, is capable of synthesizing purine and pyrimidine nucleotides from phosphoribosyl pyrophosphate (PRPP), hypoxanthine and thymidine. The hybridoma cell will survive in HAT medium, however, the NS-1 cell, an 8-azaguanine resistant cell line lacking HPRT, will eventually die out due to deficient purine in nucleotide synthesis. Because splenocytes have limited growth potential in culture medium, the splenocyte : splenocyte cells will die out within two weeks. Therefore, the NS-1 : splenocyte fused hybridoma clones can be selected by HAT medium.



The average antibody concentration obtained from *in vivo* ascites production was 0.48 mg/mL, which is much lower than the expected range of 1-10 mg/mL. However, the concentration was certainly far higher than the reported average antibody concentration obtained from *in vitro* production [53]. The M1G9 clone does not produce ascites well, but tends to form a huge solid peritoneal tumor. Although some antibodies could be harvested from ascites production, high recoveries were obtained from the abdominal washing solution and the solid tumor extraction. This indicates that the antibody production *in vivo* by ascites technique was not successful. Instead of pristane, some reports [53] suggest that Incomplete Freund's Adjuvant (IFA) can promote better ascites production. To prevent the formation of solid tumors, the multiple sites' injection of hybridomas in mouse abdomen and serial passing the ascites from the previous ascites-producing mouse to another just primed mouse have been found to be useful.

For the preparation of HRPO conjugated CK-M antibodies, the sugar groups of HRPO were first oxidized by sodium periodate at acidic pH (pH 4.0). After activation, the HRPO was incubated with CK-M antibody at pH 8.3, then the intermediate, a Schiff's base or a carbinoamine, formed in this process and was reduced by sodium borohydride. This generates a stable covalent bond between CK-M antibody and HRPO. A possible scheme is illustrated as following [69].



CK-B antibody B1D10 was shown to be positive against CK-BB isoenzyme and negative against CK-MM in the initial regular and optimized screenings, but was not reproducible in the optimized screening later. This may be due to the denaturation of the CK isoenzymes, which caused the alteration of their tertiary conformations. The recognizable epitopes could be changed and the affinity between B1D10 antibody and CK-BB isoenzyme could be reduce. This could be the major obstacle in the research.

C. ELISA Condition Study

The adsorption of antibody to the solid phase and the system equilibrium in antigen-antibody incubation are two of the most important factors in this ELISA system. Of these, the physical adsorption and the antibody-antigen reaction are two different processes: the former is a nonspecific, noncovalent and irreversible process; and the latter is a specific, noncovalent, and reversible equilibrium [31]. CK-B antibody was used as the antibody for solid phase immobilization and CK-M antibody was prepared as the second antibody for HRPO conjugation. In the first step of the assay, CK-MB isoenzyme was bound to the immobilized CK-B antibody to form immobilized CK-B mAb–CK-MB complexes. This is a simple bimolecular equilibrium in which hydrogen bonding, van der Waals forces, and electrostatic and hydrophobic interactions are involved. However, the equilibrium between the immobilized CK-B mAb–CK-MB complex and the free HRPO conjugated CK-M antibody (CK-M mAb-HRPO) is more complicated. This is because the multimeric binding between CK-B as well as CK-M antibodies and CK-MB isoenzyme stabilizes the final products, the immobilized CK-B mAb–CK-MB–CK-M mAb-HRPO complex.

Generally, the binding constant of an epitope to a monoclonal antibody ranges from 10^8 M^{-1} (low affinity) to 10^{12} M^{-1}(high affinity) [53]. High-affinity antibodies usually have better performance characteristics in immunochemical measurement than low-affinity antibodies because of their higher antigen binding capabilities and the

more stable antibody-antigen complexes. The overall interaction is a balance of many attractive and repulsive interactions at the interfaces of antibodies and epitopes of the antigens. A small change in antigen structure can profoundly affect the strength of the antibody-antigen interaction. Because CK-MB is a unstable enzyme, the binding affinity between antigen and antibody would be affected by the binding epitope conformation change on the degraded CK-MB isoenzyme. This theory supports the results from the antibody screening assay.

The pairing of CK-B antibody and CK-M antibody for the CK-MB detecting ELISA system was decided by the binding affinities of antibodies as well as the relative stereo-conformations of two antibody binding orientations on the antigen. The latter is more important. In the CK-MB detecting ELISA system, M1D6 showed the highest CK-M subunit binding affinity (Fig.13) and, fortunately, the best epitope binding orientation (Fig. 18).

After the initial adsorption of antibody, a blocking agent was used to block any unbound sites on the surface of solid phase that may cause nonspecific binding of other contents in the sample. The blocking agent was required to possess high purity and immunological inertness. The results of the blocking studies (Fig. 22, 23 & Table 5) indicated a possible cross-reaction of OVA with mouse IgG as illustrated by its high background that eventually lowered the signal to noise ratio. Different Sigma BSA product (A-2153, A-3059, A4503, A-7030, and A7906) are provided due to different purities and ingredients from different preparations. BSA (Sigma A-3059, 98-99%

purity) was found to provide better blocking effect and assay performance than other BSA lots. BSA Sigma A-4503, the original blocking agent used in all of the preliminary studies for well performance, has 96% purity with most globulins. This impurity, globulins, could induce nonspecific binding in the ELISA assay and increase the assay background reading.

Owing to the unstable properties of CK-MB and HRPO, the incubation conditions, time and buffer, are critically important to ELISA performance. It was reported [19] that some thiol groups are located near the active site of CK isoenzymes and are important to the catalytic function of the enzyme. The oxidation of these active thiol groups would change the tertiary conformations of CK isoenzymes and thus altered their enzymatic activities and integrity of the antibody recognized binding epitopes, if the epitopes are located close to the area. The use of incubation buffer, containing 1 mM reduced glutathione and 0.1% BSA in PBS, seems to stabilize the recognized epitopes on CK-MB. Similarly, horseradish peroxidase, a light and temperature sensitive enzyme, was also stabilized by the use of the incubation buffer, 0.1% BSA in PBS.

D. Performance Study

In the assay, a narrow CK-MB detecting range, 7.3-15 ng/mL, demonstrates the low affinity binding between B1D10 and the epitope on the CK-B subunit of CK-MB. This range covers twice of CK-MB normal range in human serum, 1-8 μg/L. The

12.3% C.V. of the within-batch precision reflected the operating difficulty of thorough washing for maintaining the weak B1D10–CK-MB binding. The partial cross-reaction between B1D10 and CK-M subunit also was observed in this assay. From a study of adding 100 ng/mL CK-MM into the incubation of a CK-MB detecting linearity study, the results obtained clearly revealed the cross-reactive effect of CK-MM on the linear range of CK-MB (data not shown). The CK-MB detecting ELISA showed a 4.8% of cross-reactivity with CK-MM as compared to CK-MB at similar concentration of 100 ng/mL (Fig. 34). These observations make the clinical validation study of the ELISA system invalid, and clearly indicate that a better quality CK-B monoclonal antibody, in terms of affinity and specificity, should be generated for this system.

E. Summary

. Recognition of structural determinants rather than measurement of enzymatic activity would be more significant to the clinical diagnosis. This ELISA system estimating the existing amount of CK-MB in a sample is based on a CK antibody-recognized conformation, but not on CK enzyme activity. However, CK isoenzymes are unstable proteins, as well as 77.77% of coding region homology and 77-82% amino acid sequence homology exist between CK-BB and CK-MM [9, 29]. The possibility to generate specific hybridoma clones is low. Including the consideration of the two main problems for optimizing this ELISA system: the stable antibody-recognized epitopes and antibodies with high CK-MB affinity. This research becomes

more difficult than what was expected in the beginning.

This research provided a good model and important studies for the development of a CK-MB detecting ELISA system. The clinical validation can be achieved by a CK-B antibody with high affinity and specificity to a conformationally stable antigenic epitope. Once a CK-MB detecting ELISA system with better sensitivity and detection range has been achieved, the clinical diagnostic kit can be expected to be developed in the future.

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