Peroxidase-Coupled Method for Kinetic Colorimetry of Total Creatine Kinase Activity in Serum

Michael C. Wimmer, Joseph D. Artiss, and Bennie Zak

We describe a peroxidase-coupled method involving a colorimetric indicator reaction for determining the total activity of creatine kinase (EC 2.7.3.2) in serum. The kinetically favorable reverse reaction is exploited to generate adenosine 5'-triphosphate, which is used in the glycerol kinase-catalyzed phosphorylation of glycerol. The glycerol 3-phosphate so generated is oxidized in the presence of α-glycerophosphate oxidase to produce hydrogen peroxide, which is reduced in the presence of peroxidase with the simultaneous oxidation and coupling of 4-aminoantipyrine and 2-hydroxy-3,5-dichlorobenzenesulfonate to produce an intensely colored red chromogen. Results of the proposed method (y) correlate well with those of the Boehringer-Mannheim "CK-NAC UV" method as applied to the Hitachi 705 chemistry analyzer (y = 1.025x - 18.1, r = 0.9985, n = 100, range = 19-4531 U/L). The sensitivity of the method, based on molar absorptivities, is nearly fourfold that of procedures involving the reduction of NADP+.

Additional Keyphrases: coupled enzymic assays · detection of myocardial infarction · measurement of ATP, glycerol

Creatine kinase (CK, EC 2.7.3.2) catalyzes the reversible phosphorylation of creatine. The activity of CK in serum has been used for many years as a sensitive in-vitro indicator of skeletal muscle disease and myocardial infarction. Numerous analytical techniques have been developed for quantifying CK activity in serum, the more popular ones being based on the method of Oliver (1) and its later modifications (2-5). These methods are based on the kinetically favorable reverse reaction in which ADP is phosphorylated to ATP by creatine phosphate, which is monitored by the reduction of NADP+ at 340 nm.

Although methods based on this reaction sequence are well established and work well, they retain certain inherent limitations. They suffer significant interference from lipo- mia (light-scattering effects are tremendously enhanced at wavelengths below 400 nm), their sensitivities are limited by the relatively low molar absorptivity of NADPH, and they are plagued by high reagent blanks.

Recently, Findlay et al. (6) utilized a novel reaction sequence in a "dry film" quantification of CK activity in serum. This technique reportedly worked well, but it cannot be applied to conventional instrumentation, which requires aqueous reagents.

We have utilized a similar technique in developing a peroxidase-coupled reagent for the kinetic determination of total serum CK activity in aqueous media. Unlike existing CK methods involving ultraviolet spectrophotometry, our procedure allows for the use of a Trinder-type (7) peroxidase-catalyzed oxidation system as the indicator reaction. Our reaction scheme, with a coupling reagent for the indicator reaction differing from that in the Findlay procedure, is as follows:

Creatine phosphate + ADP \(\xrightarrow{\text{creatine kinase}}\) creatine + ATP

ATP + glycerol \(\xrightarrow{\text{glycerol kinase}}\) glycerol 3-phosphate + ADP

Glycerol 3-phosphate + O\(_2\) \(\xrightarrow{\text{glycerophosphate oxidase}}\) dihydroxyacetone phosphate + H\(_2\)O\(_2\)

2 H\(_2\)O\(_2\) + 4-aminoantipyrine + HDCBS \(\xrightarrow{\text{peroxidase}}\) red chromogen

The development of this reagent system offers several advantages over the currently available methodologies, the most important being a nearly fourfold increase in sensitivity. Increasing the wavelength of measurement from 340 to 510 nm minimizes the light-scattering effects of turbidity. The high reagent blank absorbances have been eliminated and additional improvements in peroxidase-coupled chromogens may further increase the assay sensitivity.

Materials and Methods

Apparatus: We measured CK activities with a Model 34 spectrophotometer and recorder (Beckman Instruments Inc., Fullerton, CA 92634) thermostatically maintained at 37 °C.

Materials: Glycerol kinase (from Streptomyces caesu; AT&F; glycerol 3-phosphotransferase; EC 2.7.1.30), L-α-glycerophosphate oxidase (from Aerococcus viridans; glycerol-3-phospho-O\(_2\)-oxidoreductase; no EC no. assigned), and peroxidase (from horseradish; donor:H\(_2\)O\(_2\) oxidoreductase; EC 1.11.1.7) were obtained from Ferment Biochemicals, Inc., Elk Grove Village, IL 60077.

AMP (from yeast), and P\(_1\).P\(_2\)-di(adenosine-5')pentaphosphate (DAPP) were all obtained from Sigma Chemical Co., St. Louis, MO 63178.

The following reagents were obtained from Research Organics, Inc., Cleveland, OH 44124: sodium 2-hydroxy-3,5-dichlorobenzenesulfonate (HDCBS); 2-bis[2-hydroxyethyl]amino]ethanethiolacetic acid (BE8); 3-(N-morpholino)-2-hydroxypropanesulfonic acid (MOPSO); 1,4-piperazinediethanesulfonic acid (Pipes); 1,3-bis(tris(hydroxymethyl)methylamino)propane (Bis-Tris propane).

Reagents: Prepare the CK reagent in imidazole/HCl buffer, 100 mmol/L, pH 6.8, to contain the following concentrations per liter: 4-aminoantipyrine, 1.5 mmol; MgCl\(_2\), 10

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mmol; glycerol, 0.5 mmol; ADP, 3 mmol; AMP, 5 mmol;
creatine phosphate, 30 mmol; DAPP, 10 μmol; glycerol ki-
nase, 1 kU; glycophosphate oxidase, 10 kU; and peroxi-
dase, 5 kU.

Prepare the hdcbs reagent in imidazole/HCl buffer, 100
mmol/L, pH 6.8, to contain 15 mmol of hdcbs and 10 μmol
of DAPP per liter.

Prepare the glutathione reagent at 40 mmol/L in imida-
zoles HCl, 100 mmol/L, pH 6.8.

All reagents were prepared freshly and were stable for
at least one 8-h working day.

Determination of molar absorptivity: We prepared a
solution of ATP to contain approximately 1 mmol/L, then
standardized it by quantitatively reacting glucose and
NADP+ with catalytic amounts of hexokinase (EC 2.7.1.1)
and glucose-6-phosphate dehydrogenase (EC 1.1.1.49). We
added 1 μL of glutathione reagent to 20 μL of serum, incubated at 37 °C for 10 min, then mixed in 0.9
mL of CK reagent and 0.1 mL of hdcbs reagent. The CK
reagent had been prepared without phosphocreatine or
ADP, to prevent generation of ATP from endogenous CK
or adenylate kinase (EC 2.7.4.3). We then added 10 μL of 1.034
mmol/L ATP solution to the above mixture, incubated at 37
°C for 5 min, then measured the absorbance at 510 nm.
Six replicate determinations gave an average absorbance of
0.220 A, corresponding to an effective molar absorptivity
of 21,900 L mol⁻¹ cm⁻¹.

Procedure: Pipet 20 μL of serum into the bottom of a 12 ×
75 mm test tube. Add 1 μL of glutathione reagent directly to
the sample, vortex mix, and incubate for 10 min in a water
bath set at 37 °C. Add 0.9 mL of CK reagent and 0.1 mL of
hdcbs reagent, both preheated to 37 °C. Vortex mix, transfer
the contents to a cuvette, and monitor the absorbance at 510
nm vs a reagent blank without glutathione for approximately
6 min. Obtain absorbance values from the linear portion of
the curve (after the 2–3 min lag phase). Calculate serum
CK activity (U/L) as follows:

\[
CK = \frac{\Delta A/min}{21,900} \times \frac{1.021}{0.02} \times 10^6 \times 2
\]

where 21,900 = effective molar absorptivity; 1.021 = total
reaction volume; 0.02 = sample volume; 10^6 converts moles to
micromoles; 2 = two molecules of H₂O₂ consumed per
chromogen molecule formed.

One unit of activity is conventionally defined as that
amount of enzyme required to convert 1 μmol of creatine
phosphate to creatine per minute at 37 °C under the
described conditions.

We found the assay to respond linearly with concentration
to at least 2000 U/L for a 20-μL sample and 1.0 mL of
reagent. Specimens with CK activities exceeding 2000 U/L
should be reassayed after dilution with physiological saline.

Note: To facilitate possible automation, nine parts of CK
reagent may be premixed with 1 part of hdcbs reagent
immediately before use. One milliliter of combined reagent
is then added to the reactivated serum and monitored as
described above. Also, it is probably not necessary to moni-
tor the linear portion of the curve for the 3–4 min that we
describe.

Samples: All samples were freshly drawn (within 24 h)
serum specimens from the clinical laboratories of Detroit
Receiving Hospital/University Health Center. The samples
were preselected to include an adequate range of activities
for comparison.

Results

We initially optimized the pH by using a 100 mmol/L
imidazole HCl buffer. Duplicate assays of a pool of six
patients’ sera demonstrated a relatively broad pH optimum
from about 6.6 to 7.0, with maximum activity at pH 6.8
(Figure 1a). We also evaluated six other buffers with pKₐ
values at or near the determined pH optimum of 6.8. The
results of this evaluation (Table 1) revealed that at pH 6.8
and 100 mmol/L, imidazole, HCl, MES, and MOPS gave
virtually identical CK activities; Mops slightly less; and
ACES, TRIS, and Bis-Tris propane substantially lower results.

Because of the relatively low cost and general availability
of imidazole, we decided to use imidazole HCl as the
buffering system in all further work. The optimal creatine
phosphate concentration was between 30 and 60 mmol/L
(Figure 1b). ADP concentrations from 3 to 4 mmol/L yielded
identical results (Figure 1c).

Maximum CK activity was found over a wide range
of glycerol concentrations (Figure 1d). Increasing the glycerol
concentration to as much as 10 mmol/L had no inhibitory
effects on the assay. Therefore, high concentrations of en-
dogenous glycerol will not interfere. The requirement of CK
for magnesium ion was found to have a broad optimum from
8 to 20 mmol of MgCl₂ per liter (Figure 2a). The substitution
of magnesium acetate yielded similar results, and it has
the advantage of being less hygroscopic and thus easier to
handle. Maximum CK activity was obtained over a wide

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<th>Table 1. Buffer Evaluation</th>
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</tr>
<tr>
<td>Imidazole HCl</td>
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<td>Bis-Tris propane</td>
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*Each at 100 mmol/L, pH 6.8.

**Average of duplicate assays performed on pooled sera from six patients.

*CK activity was maximum (= 100%) in imidazole HCl buffer.
Fig. 2. Optimization data for (a) magnesium chloride, (b) glycerol kinase, (c) α-glycerophosphate oxidase, (d) peroxidase.

range of glycerol kinase activities, with as little as 0.5 kU being required per liter (Figure 2b). Likewise, maximum CK activity was found over a very wide range of peroxidase activities (Figure 2d). Increasing the peroxidase concentration to 200 kU/L had no inhibitory effects. However, a pronounced blank reaction is observed at peroxidase concentrations above 5 kU/L.

Various commercial vendors define their units differently. For our optimization we used an enzyme that decomposes 1 μmol of H₂O₂ per minute at 25 °C.

Although a wide variety of Trinder-type coupling reagents might be used in this system, we elected to use 4-aminoantipyrine and H₂Cubes. They are, to our knowledge, the most sensitive pair available, and we have successfully adapted them to a number of analyte systems (8–12). Maximum CK activity was obtained at co-substrate concentrations of 1.0 to 1.5 mmol/L. Concentrations greater than 1.5 mmol/L were found to be increasingly inhibitory.

CK is rapidly inactivated in serum but may be reactivated by the addition of various sulphydryl compounds (13). Although a wide variety of reactivating thiol agents have been described, we limited our study to glutathione, N-acetylcysteine (NAC), and cysteine. We found that glutathione provided maximum reactivation of CK in our system. We reactivated CK by incubating serum with glutathione, 40 mmol/L, for 10 min at 37 °C. Results of the optimization of glutathione concentration are shown in Figure 3. Reactivation of serum for less than 10 min decreased the CK activity. Glutathione in concentrations >40 mmol/L inhibited the final color formation. This inhibition takes place at the level of the indicator system rather than through inhibition of CK activity, as we demonstrated by adding constant amounts of hydrogen peroxide to solutions of peroxidase, 4-aminoantipyrine, and H₂Cubes and noting that the color intensity decreased with increasing concentrations of glutathione. This is not totally unexpected, because peroxidase-coupled reactions are redox indicator systems and glutathione functions as a reducing agent. If present in excess of that required to reactivate CK, glutathione will tend to maintain the indicator reactants in their reduced, colorless state. The reducing nature of the thiol group also dictates that it be added directly to the serum sample for maximum CK reactivation. If glutathione is included in the working reagent in concentrations sufficient for CK reactivation, chromogen formation is totally inhibited.

Considerable work has been done in recent years on the effect of chelators on CK activity (14–17). We evaluated the addition of various concentrations of ethylenediaminetetraacetic acid or ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid to the CK reagent. Their incorporation at concentrations found beneficial in previous studies (i.e., up to 2 mmol/L) produced no additional increase in CK activity over the reagent prepared without chelator. The reason for this discrepancy is not readily apparent. However, Szași et al. (15) have proposed that chelators may have a protective effect on reagent thiol groups. Removal of the thiol from the CK reagent may protect it from oxidation by reagent constituents, thus explaining the ineffectiveness of the chelators in this formulation.

Adenylate kinase, when present, will falsely increase apparent CK activity by generating ATP from ADP. Historically, AMP (1–3), and later fluoride ions (4), have been included in reagents as adenylate kinase inhibitors. However, considerable attention has been given recently to the potent adenylate kinase inhibitor P₁,P₄-di(adenosine-5') pentaphosphate (18, 19). We have used the recommended combination of AMP and DAPP at concentrations of 5 mmol/L and 10 μmol/L, respectively.

Bilirubin has been well studied as an interference in peroxidase-coupled reactions (20, 21). We evaluated the effects of bilirubin in two ways: We added known quantities of bilirubin to a serum pool with a known average CK activity of 411 U/L. Even at an added bilirubin concentration of 200 mg/L, CK activity was depressed by only 5.8%. We also assayed 10 icteric samples for CK activity with both the "CK-NAC UV" and proposed procedures (Table 2). Over

<table>
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<tr>
<th>Total bilirubin</th>
<th>Direct bilirubin</th>
<th>CK-NAC UV</th>
<th>Predicted CK*</th>
<th>Proposed CK*</th>
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*a Reference method.
*b Calculated from comparison data with reference method (y = 1.025x – 18.1).
*c Values determined with proposed procedure.
Fig. 4. Correlation between results by BMD "CK-NAC UV" kit used in the Hitachi 705 (x) and by the proposed method (y) in the range studied, 46–141 mg of bilirubin per liter, all of the CK values fell within the predictive interval (22) of the line of regression (see below) at a 95% confidence level.

We compared this assay (y) with the Boehringer Mannheim CK-NAC UV procedure as applied to the Hitachi 705 chemistry analyzer (x) (Figure 4). The CK-NAC procedure is an application of the IFCC Reference Method and is based on the optimized conditions of Szasz et al. (3). Sera with CK activities ranging from 19 to 4551 U/L were preselected, so as to include a broad range of activities. Linear regression analysis performed on data for 100 samples provided the equation: \( y = 1.025x - 18.1; \) \( S_y = 31.7; r = 0.9985. \) This correlation would seem to suggest that the establishment of a separate norm range for this procedure is unnecessary. Table 3 presents our precision data for the proposed assay in which we used pooled sera at three levels of CK activity.

**Discussion**

CK assays that exploit the kinetically favorable reverse reaction must couple the initial reaction via creatine or ATP. Previous colorimetric methods for CK in which the reverse reaction was used have generally coupled through creatine (23–25). Unfortunately, these methods were either technically demanding, poorly sensitive, or too nonspecific (26). In the present procedure these problems have been largely avoided.

Perhaps the greatest merit of this assay is its increased sensitivity as compared with conventional methods. This is made possible through a unique reaction sequence in which the peroxidase-catalyzed oxidation of a Trinder-type indicator is used. The effective molar absorptivity is 21 900 L mol\(^{-1}\)cm\(^{-1}\). This is nearly four times that of systems involving NADPH as an indicator, which has a molar absorptivity of only 6220 L mol\(^{-1}\)cm\(^{-1}\) (27). We anticipate that this increased sensitivity will prove advantageous when the proposed method is used to quantify CK-MB activities separately.

Although the Trinder-type chromogen has nearly quadrupled the sensitivity of the assay, it requires that the sample be pretreated with a sulfhydryl reagent to reactivate CK effectively. This pretreatment is necessary because incorporation of the thiol into the reagent severely inhibits color formation. Autocoupling of the indicators will take place if there is too much peroxidase activity in the reagent. We recommend separation of the indicators to prevent coupling during prolonged storage of the reagent.

In summary: we have developed a kinetic assay for creatine kinase involving an indicator reaction in the visible spectrum. This method has resulted in a near fourfold increase in sensitivity over conventional methodologies. This improved sensitivity may be beneficial in the quantification of CK-MB isoenzymes. The system is flexible in that further improvements in the sensitivities of Trinder-type indicators may be easily incorporated into the reagent system. Finally, we believe that with minor procedural modifications, the proposed method may be utilized to quantify ATP or glycerol.

**Table 3. Reproducibility Study**

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References

15. Szasz G, Waldenstrom J, Gruber W. Creatine kinase in serum: 6. Inhibition by endogenous polyvalent cations, and effect of chela-