# A CONTINUOUS METHOD FOR MEASURING CALPAIN ACTIVITY

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Casein is the most widely used substrate for measuring calpain activity. In caseinolysis assay, the substrate has to be separated from the hydrolysates by TCA precipitation and centrifugation. The TCA-soluble hydrolysates were then determined by measuring the absorbance at 278 nm (Waxman, 1981), by dye-binding method (Jiang et al., 1991), or by fluorescamine binding method (Melloni et al., 1984). Buroker-Kilgore and Wang (1993) reported a modified caseinolysis assay to measure calpain activity, which employed Coomassie brilliant blue G-250 to interact with casein but not the hydrolysates. This paper aimed to develop a rapid and reliable measurement for calpain activity without prior separation of substrate from hydrolysates, which can be used in continuous ELISA measuring system.

**Methodology:** Calpain II and calpastatin were extracted from bovine muscle. A Pharmacia Hiload and FPLC systems were used for all column chromatography. Calpain II and calpastatin were purified by a series of DEAE-Sepharose Fast Flow, Sephacryl S-300 HR, Sephacryl S-200 HR, Q-Sepharose HP and Red-120 Agarose columns. Casein Assay for Calpain Activity: Calpain in 50 mM imidazole-HCl buffer (pH 7.5) containing 10 mM β-Me, 1.0 mM $NaN_3$ and 4 mg/mL of casein was pre-incubated at 25°C for 5 min. Then $CaCl_2$ was added to a final concentration of 15 mM to initiate the calpain hydrolysis reaction. Time scanning at wavelength of 400~600 nm was immediately performed at 25°C. The maximum velocity was determined from the changes in $A_{500}$ with the reaction time. One unit of the calpain activity is defined as the amount of proteinase that caused an increase of one absorbency unit at 500 nm after a 10 min incubation at 25°C. Casein-TCA Assay for Calpain Activity: For comparison the feasibility of the developed method with the typical measurement, casein-TCA assay was also employed. The reaction mixture consisted of 4 mg/mL of casein, 50 mM imidazole-HCl (pH 7.5), 10 mM β-Me, 1 mM $NaN_3$, 5 mM $CaCl_2$ and appropriate amount of calpain. After 30 min incubation at 25° C, equal volume of 10% TCA was added to stop the reaction and centrifuged at 3000 rpm for 15 min. The absorbance of supernatant at 278 nm were measured. One unit of calpain activity was defined as the amount of enzyme that caused an increase of one absorbency unit at 278 nm after 30 min incubation at 25°C and corrected by subtracting the absorbance of blank, which used 5 mM EDTA instead of 5 mM $CaCl_2$. Calpastatin Assay: One unit of inhibitory activity was defined as the amount of calpastatin which could inhibit one unit of calpain II.

**Results and Discussion:** No absorbance was observed on the reaction mixture of control, which used 5 mM EDTA instead of 5 mM $CaCl_2$. This phenomenon suggested that the reaction mixture for measuring the calpain hydrolysing activity does not cause any turbidity. However, the maximum absorbance of the reaction mixture shifted from 490 to 545 nm during 10.5 min reaction at 25°C when the 5 mM $CaCl_2$ was added to activate calpain. The maximum absorbance of the hydrolyzed products during 2~7 min reaction was observed at around 500 nm and increased with the increase of reaction time (Fig. 1). This absorbance ($A_{500}$) was, therefore, used on the following experiments to evaluate the feasibility for measuring the calpain activity. The time course of casein proteolysis by calpain revealed a sigmoid curve (Fig. 2). Since this calpain hydrolyzing reaction showed an initial lag of product formation (Fig. 2), the calpain might be a hysteretic proteinase. **Effect of enzyme concentration:** The $A_{500}$ of the reaction mixture with different amounts of calpain II reached to a constant level and then maintained at that levels (Fig. 2). The time for rising to the constant level varied with the amounts of calpain added. The higher the calpain added, the faster the maximum absorbance reached. However, after the $A_{500}$ reached to highest level, it decreased slightly after 30 min reaction (data not shown). The lag time decreased rapidly with the increase of calpain II (Data not shown). Since the lag time represents the extrapolated values of the hydrolysates formed on the linear region of curves, with an appropriate amounts of calpain, the reaction could reach to a maximum steady velocity within 3 min (Fig. 2). The maximum velocity increased linearly with the increase of calpain II between 10 and 60 μg. This phenomenon was similar to that measured by casein-TCA assay (Fig.3). Accordingly, one unit of calpain activity was, in this study, defined as the amount of enzyme that caused an maximum velocity increase of one absorbance unit at 500 nm within 1 min reaction ($\Delta A_{500}$/min) at 25°C. The minimum requirement of casein for this methodology was greater than 2 mg/mL. **Calcium requirement:** Using traditional caseinolysis assay, the calpain II from bovine muscle was half-maximally activated by 0.25 mM calcium ions. However, calcium requirement for half-maximal velocity was about 6 mM in this experiment (data not shown). According to the Superose 12 gel filtration profile of the hydrolysates, a high molecular weight (MW) protein peak appeared, however, it disappeared in the presence of

10 mM β-Me and 5 mM EDTA (Fig. 4). These data suggested that the high MW protein peak might be derived from the aggregate of hydrolysates, which might be caused by disulfide bond or cross-bridge by $Ca^{2+}$. **Effect of calpastatin concentration:** The calpain II was inhibited by calpastatin. The lag time increased gradually with the increase of calpastatin. The maximum velocity decreased gradually with the increase of calpastatin. This data was similar to that of $A_{278}$ in casein-TCA assay.

In the traditional caseinolysis assay, casein was incubated with calpain followed by precipitation of remaining protein substrate using TCA. The $A_{278}$ was used to evaluate the acid-soluble peptide. However, the developed method is a continuous assay designated for the automatic determination of the calpain activity. The maximum velocity was dependent on the enzyme amount, calcium ions and inhibitor.

**References**


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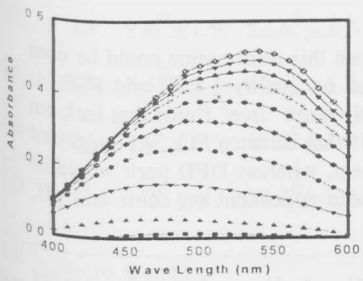


Fig. 1. The absorption spectrum for calpain II assay using casein as substrate. Absorption spectrum from 400 to 600 nm was obtained at 25°C. The scanning speed was 40 nm/sec.

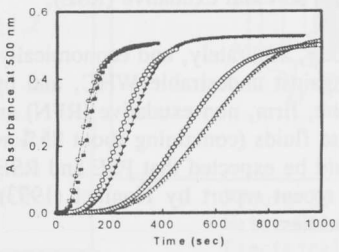


Fig. 2. Time course of calpain II assay. The reaction mixture contained 4 mg/mL casein, 50 mM imidazole-HCl buffer, pH 7.5, 10 mM β-Me, 0.5 mM $NaN_3$, and 5 mM $CaCl_2$; and was incubated at 25°C.

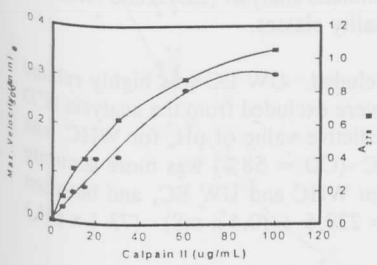


Fig. 3. Effect of calpain II concentration on activity.

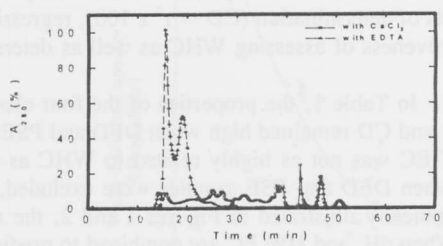


Fig. 4. Chromatographic separation of hydrolyzed products of casein by calpain II using Superose 12.