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SHORT REPORTS

ACTIVE SITE TITRATION OF THE SERINE PROTEASE CUCUMISIN FROM *CUCUMIS MELO*

MAKOTO KANEDA, YOSHIHIRO MINEMATSU*†, JAMES C. POWERS* and NAOTOMO TOMINAGA

Department of Chemistry, Faculty of Science, Kagoshima University, Korimoto, Kagoshima 890, Japan; *School of Chemistry, Georgia Institute of Technology, Atlanta, GA 30332, U.S.A.

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Key Word Index—*Cucumis melo*; Cucurbitaceae; melon fruit; protease; titration of cucumisin.

Abstract—The amount of active enzyme in cucumisin solution was determined by titration with *N*-acetyl-L-alanyl-L-alanyl-α-azaalanine *p*-nitrophenyl ester. When azapeptide was added to cucumisin, *p*-nitrophenol was rapidly released and deacylation was slow. The cucumisin used was found to be approximately 94% active.

INTRODUCTION

In general, the active enzyme in a protease preparation or in a reaction mixture is not easily assayed. Naturally occurring protease inhibitors are commonly used for such an assay. However, trypsin can be titrated by *p*-nitrophenyl-*p'*-guanidinobenzoate, releasing an amount of *p*-nitrophenol that is exactly equal to that of the active trypsin molecule. This was verified spectrophotometrically [1]. This spectrophotometric titration is useful because of its convenience, accuracy and rapidity. But little is known concerning good titrants for other proteases.

Powers *et al.* recently demonstrated that new azapeptide *p*-nitrophenyl esters could be utilized as active site titrants for chymotrypsin-like enzymes and elastases [2, 3]. Cucumisin (EC 3.4.21.25) extracted from the Prince melon by Kaneda *et al.* is a serine protease [4] and its four amino acid sequence around the reactive serine residue, Gly-Thr-Ser-Met, is identical with that of subtilisin [5]. Until now, the active site of cucumisin could not be titrated. However, now it has become possible to titrate the active site of cucumisin by Ac-Ala-Ala-Aala-ONp.

RESULTS AND DISCUSSION

When the azapeptide was added to the cucumisin solution, it was observed that the release of *p*-nitrophenol took place rapidly and deacylation slowly (Fig. 1). The active enzyme concentration can be calculated from the release of *p*-nitrophenol ($[E_0] = \Delta A_{345}/\varepsilon_{HONp}$, $\varepsilon_{HONp} = 6250$ at pH 6.0). The turnover rate k_{cat} is then

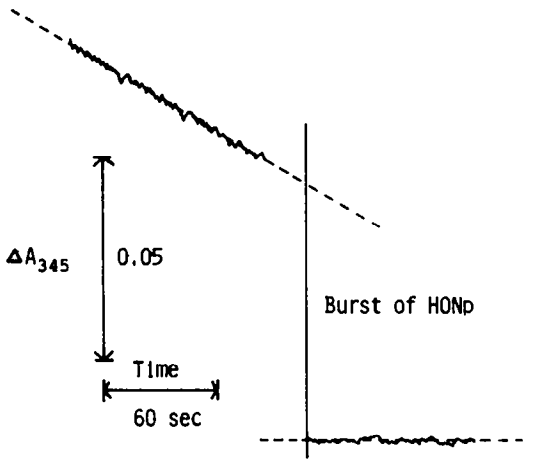

Fig. 1. Reaction of cucumisin with Ac-Ala-Ala-Aala-ONp in 0.1 M citrate buffer at pH 5.0, 25°. The reaction mixture consists of 208 μM Ac-Ala-Ala-Aala-ONp, 10 μM concentration of cucumisin. The active enzyme concentration (E_0) and turnover rate (k_{cat}) were calculated from the burst and slope and had the values of 9.4 μM and 1.8×10^{-3} sec^{-1}.

†Present address: Department of Chemistry, Faculty of Science, Kyushu University, Higashi-ku, Fukuoka 812, Japan.

$(\Delta A_{345}/s)/\Delta A_{345}$ (burst). Table 1 shows the results obtained from the reaction of azapeptide with cucumisin. The acylation reaction had a 1 : 1 stoichiometry with respect to enzyme concentration based on the release of p-nitrophenol. The turnover rate (k_{cat}) was pH-dependent and increased by a factor of 6 upon going from pH 5 to pH 6. The cucumisin used here was found to be approximately 94% active. This enzyme preparation was lyophilized in the presence of 30% of sucrose, while sucrose-free preparations were below 70% active.

Table 1. Reaction of cucumisin with Ac-Ala-Ala-Aala-ONp

pH	[I] (μM)	$[E]^{*}_{280}$ (μM)	$[E]^{\dagger}_{0}$ (μM)	% Purity‡	$k_{cat} \times 10^4$ (sec^{-1})
5.0	208	10	9.4	94	18
5.5	208	10	9.5	95	57
6.0	208	10	9.3	93	110

*The enzyme concentration based on A_{280}.

†Enzyme concentration determined from the burst (ΔA_{345}) of HONp.

‡$100 \times [E]_0/[E]_{280}$.

EXPERIMENTAL

Cucumisin was isolated from Prince melon, *Cucumis melo* L. var. Prince, by the method of Kaneda *et al.* [4]. Azapeptide was synthesized by the method of Gupton *et al.* [2].

The reaction of cucumisin with azapeptide was carried out in a soln which contained a *ca* 20-fold excess of azapeptide over enzyme. Stock soln of azapeptide in MeCN was prepared at a concn of 2.5 mM. Cucumisin stock soln was made up in H_2O and had a concn of 120 μM. Exact enzyme concn were determined by absorbance at 280 nm ($E^{1\%}_{280} = 10.0$). Three buffers were prepared: pH 6.0, 0.1 M citrate; pH 5.5, 0.1 M citrate; pH 5.0, 0.1 M citrate. All reactions were carried out at 25° by mixing 50 μl of the azapeptide stock soln with 500 μl of the appropriate buffer in a cuvette. An identical reference sample was prepared and a baseline was recorded at 345 nm. The reaction was initiated by the addition of 50 μl H_2O to the reference cuvette followed by the addition of 50 μl cucumisin stock soln to the sample cuvette. The recorder was immediately started upon the addition of enzyme to the sample cell and the reaction rate was observed. The background hydrolysis rate at pH 6.0 of azapeptide is negligible during the measurement [3].

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