Journal of Industrial Microbiology, 3 (1988) 259–261
Elsevier
SIM 00127

Short Communication

An inexpensive insoluble chromogenic substrate for the determination of proteolytic activity

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Received 26 March 1987
Revised 17 January 1988
Accepted 20 January 1988

Key words: Casein; Chromogenic substrate, insoluble; Proteolytic activity

SUMMARY

A new insoluble chromogenic substrate for the determination of proteolytic activity was developed. This substrate was prepared by incorporating black drawing ink into casein and heating this complex at 200°C for 4 h. It is especially suitable for determining the activity of alkaline bacterial proteinases.

INTRODUCTION

Microbial proteolytic enzymes are widely used in industry. They are prepared by cultivating highly productive microbial strains. During the cultivation and purification steps the activity of desired proteinases has to be measured.

Various substrates can be used for determining proteolytic activity [1,2]. Among them, insoluble chromogenic substrates are of special interest. Most of these substrates, however, are rather expensive. For routine procedures, some inexpensive substrates can be used successfully.

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The preparation and application of such an inexpensive, insoluble chromogenic substrate is described in this paper. The prepared substrate is based on thermally modified casein, the chromogenicity of which is caused by black drawing ink. This new substrate is especially suitable for the measurement of proteolytic activity of alkaline bacterial proteinases.

MATERIALS AND METHODS

Materials

Bacto isoelectric casein was obtained from Difco, U.S.A. Casein according to Hammarsten was from Reanal, Hungary. Common chemicals were

from Lachema, Czechoslovakia. Black drawing ink for drawing pens, 'Centrograf', was obtained from KOH-I-NOOR, Dačice, Czechoslovakia. Black drawing ink (black India ink) is a suspension of the finest carbon black in a weak alkaline solution of shellac [3]. A crude lyophilized preparation of extracellular bacterial alkaline proteinase produced by an alkalophilic strain of *Bacillus* sp. (specific activity 220 000 Delft units (D.U.)/g) was obtained from the Research Institute of the Fat Industry, Rakovník, Czechoslovakia. Concentrated culture medium after cultivation of an alkaline proteinase-producing bacterial strain was obtained from a cooperative farm in Slušovice, Czechoslovakia. Mikrozym, a mixture of neutral and acidic proteinases produced by some strains of *Bacillus subtilis*, was obtained from the Department of Milk and Fat Technology, Institute of Chemical Technology, Prague, Czechoslovakia. Commercial bacterial alkaline proteinases Alcalase and Maxatase are the products of Novo Industry, Denmark and Gist Brocades, The Netherlands, respectively. Trypsin, chymotrypsin, papain and pepsin were from Léčiva and Lachema, Czechoslovakia.

Preparation of insoluble substrate

Casein (10 g) was dissolved by heating in 400 ml of 0.2% (w/v) sodium hydroxide solution. This solution was allowed to cool to ambient temperature and 5.0 ml of black drawing ink were added. The black suspension was stirred on a magnetic stirrer for 30 min. Casein was then precipitated together with black drawing ink by the dropwise addition of 30% (v/v) acetic acid. Usually 8–9 ml of acetic acid were used for complete precipitation. The black precipitate was removed by filtration, washed with 500 ml of water and transferred into a Petri dish. The precipitate was dried at 80°C and the dry material was then heated in a hot-air oven at 200°C for 4 h. The resulting thermally modified casein containing black drawing ink was then ground in a mortar and repeatedly suspended in 1–4% (w/v) sodium hydroxide until the majority of the black-coloured substances was released. The substrate was then filtered, washed with water, dried at 80°C and ground in a mortar to obtain fine particles.

Determination of proteolytic activity

Insoluble substrate (usually 20 mg) was transferred into a test tube and 2 ml of buffer (0.05 M Tris-HCl buffer, pH 8.5, containing 0.01 M $CaCl_2$) were added. The substrate was allowed to swell at working temperature (37–50°C) for 20 min and then 1 ml of sample containing proteolytic enzymes was added while the suspension was mixed. The reaction mixture was then incubated without mixing. In the standard procedure, the reaction was stopped after 30 min by filtering the reaction mixture through filter paper. The absorbance of the filtrate was measured at 400 nm in a glass 1-cm cuvette against a substrate blank (1 ml of buffer was added to the substrate suspension instead of proteinase solution). When coloured proteinase-containing solutions were used, the value of the sample blank (1 ml of proteinase solution plus 2 ml of buffer) was subtracted.

RESULTS AND DISCUSSION

Thermal modification of the casein–black drawing ink complex resulted in the formation of a black, water-insoluble material, which could be degraded by various bacterial alkaline proteinases. During the action of these proteolytic enzymes,

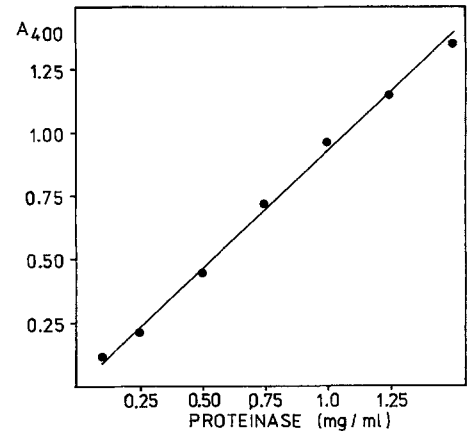

Fig. 1. Hydrolysis of thermally modified casein–black drawing ink complex as a function of concentration of alkaline bacterial proteinase (specific activity 220 000 D.U./g). The reaction mixture was incubated at 50°C for 30 min.

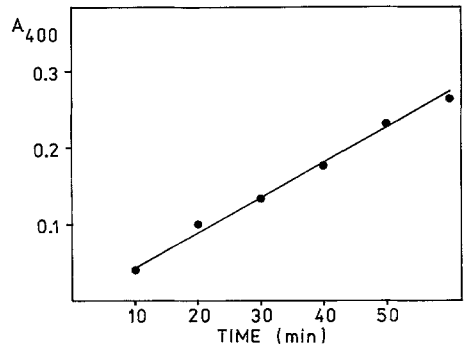

Fig. 2. Time course of digestion of thermally modified casein–black drawing ink complex by alkaline bacterial proteinase (specific activity 220 000 D.U./g; 0.1 mg/ml). The incubation was carried out at 50°C.

black drawing ink was released into the surrounding solution which became brown. The intensity of the brown colour is proportional to the proteolytic activity.

The substrate was hydrolyzed by bacterial alkaline proteinases such as Alcalase and Maxatase, and by various crude preparations of these proteinases. Trypsin, chymotrypsin and papain, as well as some other bacterial and fungal proteinases, hydrolyzed the substrate only slightly. Acid proteinases, such as pepsin and Mikrozym, and also some other microbial proteinases, did not hydrolyze this substrate.

Hydrolysis of this insoluble substrate as a function of concentration of bacterial alkaline proteinase (specific activity 220 000 D.U./g) in the range 0.1–1.5 mg/ml is shown in Fig. 1. The dependence of absorbance on the amount of proteinase was linear. The detection limit was approximately 20 D.U./ml.

The time course of digestion of insoluble substrate by the same bacterial alkaline proteinase (0.1 mg/ml) is shown in Fig. 2. An incubation time up to 60 min is suitable for the assay of proteinase, since hydrolysis was linear during this time period.

This new substrate can be prepared easily. The assay is simple, fast and reproducible; it has the advantage that no precipitation step is necessary. It can be used for monitoring enzyme activity during cultivation of bacteria which produce alkaline proteinases and for determining enzyme activity during purification. The substrate could also be used for screening microbial producers of proteinases which are capable of hydrolyzing proteins which are somewhat resistant to hydrolysis.

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