Carbohydrate Polymers 135 (2016) 199–206

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Enzymolysis of chitosan by papain and its kinetics

A-Dan Pan, Hong-Yan Zeng*, Gohi Bi Foua, Claude Alain, Yu-Qin Li

Biotechnology Institute, College of Chemical Engineering, Xiangtan University, Er-huan Road, Xiangtan 411105, Hunan, PR China

ARTICLE INFO

Article history:
Received 15 June 2015
Received in revised form 10 August 2015
Accepted 17 August 2015
Available online 21 August 2015

Keywords:
Papain
Chitosan
Enzymolysis
Response surface methodology
Kinetics

Chemical compounds studied in this article:
Chitosan (PubChem CID: 21896651)
Acetic acid (PubChem CID: 176)
Sodium acetate (PubChem CID: 517045)
Sodium hydroxide (PubChem CID:14798)
Isodium hydrogen phosphate (PubChem CID: 24203)
Monosodium phosphate (PubChem CID: 23672064)
Glucose (PubChem CID: 5793)
Sodium borohydride (PubChem CID: 22959485)
D-Glucosamine (PubChem CID: 439213)
Acetyl glucosamine (PubChem CID: 439174)
Dinitrosalicylic acid (PubChem CID: 11873)

ABSTRACT

Low molecular weight chitosan (LMWC) was obtained by the enzymolysis of chitosan by papain. Enzymolysis conditions (initial chitosan concentration, temperature, pH and ratio of papain to chitosan) were optimized by conducting experiments at three different levels using the response surface methodology (RSM) to obtain high soluble reducing sugars (SRSs) concentrations. Meanwhile, the influence of chitosan substrate concentration on the activity of papain was assessed in the experiments. The enzymolysis process was analyzed using pseudo-first-order and pseudo-second-order kinetic models and the experiment data were found to be more consistent with the pseudo-second-order kinetic model. In addition, the kinetic behavior of the enzymolysis was also investigated by using Haldane model, and chitosan exhibited substrate inhibition. It was clear that the Haldane kinetic model adequately described the dynamic behavior of the chitosan enzymolysis by papain. When the initial chitosan concentration was above 8.0 g/L, the papain was overloaded and exhibited significant inhibition.

1. Introduction

Chitosan and its derivatives have shown various functional properties and made them possible to be used in the fields of food, cosmetics, biomedicine, agriculture and environmental protection (Kumar, 2000; Felt, Buri, & Gurny, 1998; Yamada, Shibuya, Kodama, & Akatsuka, 1993; Peniche-Covas, Alvarez, & Argüelles-Monal, 1992; Shahidi & Synowiecki, 1991). Even though the high molecular weight of chitosan is known to have important functional activities, insoluble at neutral pH values and high viscosity aqueous solutions makes them difficult to use in commercial applications (Xia, Liu, & Liu, 2008; Chang, Chen, & Jao, 2007; Vikhoreva et al., 2005). Therefore, a new interest has recently been emerged on partially hydrolyzed chitosan, low molecular weight chitosan (LMWC). The LMWC can be produced by either chemical or enzymatic hydrolysis. Enzymatic methods have advantages over chemical reactions, since enzymes operate under milder conditions and are highly specificity and no glucose ring modifications. Moreover, enzymatic hydrolysis may produce LMWC products and retain their original biological propertiesm (Wu, 2011; Li et al., 2005). Besides the specific enzyme such as chitinase, chitosanase and lysozyme, chitosan also could be hydrolyzed by some non-specific enzymes including cellulase, lipase, pepsin and papain (Suwan et al., 2009; Lee, Xia, & Zhang, 2008; Roncal, Oviedo, de Armentia, Fernández, & Villarán, 2007; Liu & Xia, 2006; Kumar, & Tharanathan, 2004).

Among these nonspecific enzymes, papain, a cysteine protease, is particularly attractive because of its low cost and wide acceptance in the food industry as well as the ability to depolymerize chitosan

* Corresponding author. Tel.: +86 731 58298175; fax: +86 731 58298167.
E-mail address: hongyanzeng99@hotmail.com (H.-Y. Zeng).

http://dx.doi.org/10.1016/j.carbpol.2015.08.052
0144-8617/

efficiently. So, it could be a valid alternative to enzymolyze chitosan (Kumar, Varadaraj, Lalitha, & Tharanathan, 2004; Terbojevich, Cosani, & Muzzarelli, 1996). It is indispensable to maximum utilization of papain, in order to render the enzymatic process much more economical, but the enzymolysis of chitosan is not a simple, single chemical process. The process can be partially quantified by the reaction conditions and kinetics that may accurately measure enzymolysis rate and causes for the rate slowdown during the enzymatic process.

In this work, papain was employed to depolymerize chitosan and the enzymolysis parameters (temperature, pH, chitosan substrate concentration and papain amount) were optimized using response surface methodology (RSM). Finally, the enzymatic hydrolysis process was analyzed in terms of kinetic parameters.

2. Experimental

2.1. Materials

Papain (EC3.4.22.2, ≥99%) was purchased from Sigma-Aldrich Company Ltd. Chitosan was obtained from Sigma-Aldrich Company Ltd and its degree of deacetylation (*DD*) was 91.94%. All other reagents used in the experiment were of analytical grade and used without further purification. All solutions were made with redistilled and ion-free water.

2.2. Enzymolysis of chitosan

Papain solution (1.0 g/L) was obtained by dissolving the enzyme in PBS buffer (0.1 mol/L, pH 7.0). Stock solution of chitosan (18.0 g/L) was prepared in 0.2 mol/L acetate buffer solution and it was diluted into the concentration varied from 0 to 12.0 g/L. According to the experimental design, different concentrations of papain were added into the chitosan solution. The enzymolysis was performed by employing a shake flask method with 150 mL flask in a temperature-controlled water bath. After 50 min, the reaction was quenched by heating treatment at 100 °C for 10 min. The reaction mixture was adjusted to pH 7.0 with 0.2 mol/L NaOH, and then centrifuged at 3000 rpm for 4 min, resulting in a precipitate of low molecular weight chitosan (LMWC) and a supernatant containing both chitosan oligosaccharides and monomers. The supernatant was used for HPLC analysis and the SRSs measurement, and the precipitate (LMWC) was lyophilized and weighted. All the experiments were carried out in triplicate under the same condition and average values are reported. In the kinetic experiments, the reaction was monitored at regular intervals, and the SRSs content was also detected.

After approximation of the best conditions by "one-factor-at-a-time" method in our preliminary experiments, the response surface methodology (RSM) was used to test the effect of chitosan substrate concentration, initial pH, enzymolysis temperature and the ratio of enzyme to substrate on the SRSs concentration. The response surface methodology (RSM) is defined as a statistical method using quantitative data from an appropriate experimental design to determine and simultaneously solve multivariate equations. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions. The Box-Behnken Design (BBD) was used to design experiment and optimize one response variable namely the SRSs concentration (*Y*) from the enzymolysis of chitosan. Each independent variable was coded at three levels between −1 and +1, where the variables of the chitosan substrate concentration (*S*), pH (*P*), enzymolysis temperature (*T*) and the mass ratio of enzyme to substrate (*R*) were changed in the ranges shown in Table 1. Twenty-nine experiments were augmented with three replications at the design center to evaluate the pure error and were carried in randomized order as required in many design procedures. After reaction, the response *Y* was measured. The statistical software package Design Expert software (version 8.0.6) was used for regression analysis of experimental data and to plot response surface. The model generated during RSM implementation was validated by conducting experiment on given optimal setting. The second-order polynomial model was applied to predict the response variable (*Y*) as shown below:

Table 1
Experimental range and levels of the independent variables.

Independent variables	Symbols	Units	Code levels		
			−1	0	+1
Substrate concentration	*S*	g/L	6.0	8.0	10.0
pH	*P*		4.0	4.5	5.0
Temperature	*T*	°C	40.0	45.0	50.0
Ratio of enzyme to substrate	*R*	g/g	0.08	0.10	0.12

$$Y = \beta_0 + \sum_{i=1}^{4}\beta_i X_i + \sum_{i=1}^{4}\beta_{ii} X_i^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{ij} X_i X_j$$

where *Y* was the response value (SRSs concentration), β_0, β_i, β_{ii} and β_{ij} were the regression coefficients for interception, linear, quadratic and interaction terms, respectively. X_i and X_j were the independent variables.

2.3. Viscosimetry

Under the optimal conditions of the enzymolysis, the reaction was quenched by heat at regular intervals, centrifugal separation, and the precipitates (LMWCs) at regular intervals were freeze-dried. The viscosity of the LMWCs dissolved in sodium acetate buffer (0.2 mol/L acetic acid + 0.1 mol/L sodium acetate, pH 4.5 at 30 °C) was measured using an Ostwald's viscometer. The viscosity-average molecular weights of chitosans were calculated from the classical Mark–Houwink's equation $[\eta] = kM^{\alpha}$, where $[\eta]$ was the intrinsic viscosity of chitosan, *M* was the viscosity averaged molecular weight, and *k* and α were previously determined to be $k = 6.589 \times 10^{-3}$ and $\alpha = 0.88$ at 30 °C, respectively (Wang, Bo, Li, & Qin, 1991).

2.4. Analysis of enzymolysis products

After stopping enzymolysis reaction by heat, the supernatant was collected by centrifugation, and used for the SRSs measurement using the DNS method (Tasun, Chose, & Ghen, 1970). Using glucose as standard, DNS as color developing reagent, the content of the SRSs was indirectly determined by a U-9100 spectrophotometer (Hitachi, Tokyo, Japan) at 540 nm.

At the same time, the supernatant obtained under the optimal enzymolysis conditions was analyzed for identification of chito-oligosaccharides by HPLC on Asahipak NH_2P-250 4E (250 × 4.6 mm, Shodex, Japan). HPLC was performed with an Alliance (Waters Co. USA) instrument equipped with RI 2410 differential refractometer. The analysis was carried out at 40 °C using a mobile phase of acetonitrile/water (70: 30, v/v) and a flow rate of 1.0 mL/min. *N*-acetylglucosamine (GlcNAc), D-Glucosamine (GlcN), chitosan dimer($(GlcN)_2$), chitosan tetramer ($(GlcN)_4$) and chitosan hexamer ($(GlcN)_6$) (Seikagaku, Japan) were used as authentic standard.