Annex 9-7

Improved Performance of Amberlite IRA-904 Immobilized Glucoamylase

Santhirasegarampillai Balakumar, Vasanthy Arasaratnam and Kandiah Balasubramaniam, Kokuvil (Sri Lanka)

When the glucoamylase protein immobilized to Amberlite IRA-904 was estimated by direct (*Kjeldhal*), indirect (measuring the protein left in the supernatant) and elution methods, direct and indirect methods gave almost same results while the elution method gave a protein concentration of only 68.9 % of that obtained with the *Kjeldhal* method. When the activity of the soluble and immobilized glucoamylase were compared with different substrates, the activity yield increased with decreasing molecular size of the substrate. However when the immobilized glucoamylase containing Amberlite IRA-904 was ground, it showed increase in activity with increasing molecular weight of the substrates.

1 Introduction

An indispensable enzyme in saccharification of starch is glucoamylase, (1,4-α-D-Glucan glucohydrolyase EC 3.2.1.3). Many attempts have been made to immobilize this enzyme [1, 2]. Among the immobilization methods physical immobilization offers and advantage of easy reloading of enzyme and it is less harsh to native structure of enzyme. In this report we report the improvement of the performance of Amberlite IRA-904 immobilized glucoamylase by using substrates of low molecular sizes and by reducing particle size of the glucoamylase immobilized resin.

2 Experimental

2.1 Materials

Glucoamylase (Spiriamylase® 150L) was from NOVO Industries (Denmark). Amberlite IRA-904, soluble starch and maltose were from BDH Chemical Company (England). Dextrinized starch was prepared from starch [3].

2.2 Immobilization of glucoamylase to Amberlite IRA-904

Amberlite IRA-904 (10 g) was shaken at 150 rpm with 900 mg of glucoamylase protein (9500 U activity) at 30 °C for 1.0 h and filtered through a sintered glass funnel. One Unit (U) of glucoamylase was the amount of enzyme liberating 1.0 mg of glucose in a min.

2.3 Assay of immobilized glucoamylase

Amberlite IRA-904 immobilized glucoamylase (0.5 g) was incubated for 10 min with 15.0 ml of 2.0 % (w/v) starch – 0.01 M acetate buffer solution (pH 4.5) at 55 °C and glucose produced was estimated by glucose oxidase method [4]. For soluble enzyme, diluted glucoamylase solution (25 mg protein/7.5 ml 0.01 M acetate buffer, pH 4.5) was shaken with 15.0 ml starch – 0.01 M acetate buffer solution (4.0 %, w/v) for 10 min at 55 °C and glucose produced was determined.

2.4 Estimation of protein immobilized to Amberlite IRA-904

Protein immobilized was estimated by three different methods. In indirect method the amount of protein immobilized was considered as the difference between the added enzyme protein and the protein in filtrate obtained after immobilization. The protein was estimated by modified *Lowry*'s method [5]. Secondly protein of glucoamylase immobilized to Amberlite IRA-904 (1.0 g) was also estimated by *Kjeldhal* method [6]. In the elution method immobilized glucoamylase (1.0 g) was packed in a column and eluted with 3.0 ml of 0.01 M glycine – HCl buffer (pH 2.5). Protein content in the eluate was estimated [5].

2.5 Effect of grinding on the activity of immobilized glucoamylase

Glucoamylase immobilized Amberlite IRA-904 was gently ground in a motor with pestle. Activity of glucoamylase in 0.5 g ground form was determined with (2.0 %, w/v)

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Table 1. Protein immobilized to Amberlite IRA-904 determined by different methods.

Protein added (mg)	Protein immobilized (mg g^{-1} resin)		
	Indirect	*Kjeldhal*	Elution
60	36.4	37.0	25.0
	35.0	37.0	24.0
	37.0	35.0	26.0
Mean	36.0 ± 1.0	36.3 ± 1.1	25.0 ± 8.0
120	42.0	46.0	29.0
	42.0	47.0	28.0
	44.0	47.0	30.0
Mean	42.6 ± 1.1	46.6 ± 10.5	29.0 ± 1.0

Standard error between the means of indirect and *Kjeldhal* methods when 60 and 120 mg enzyme protein/g resin used was 0.23 and 0.28, respectively.
Standard error between the means of *Kjeldhal* and elution methods when 60 and 120 mg enzyme protein/g resin used was 12.8 and 27.2, respectively.

starch, dextrinized starch (DE 36) and maltose as described above.

3 Results and Discussion

3.1 Estimation of protein immobilized to Amberlite IRA-904 by different methods

Protein immobilized from glucoamylase solution estimated by different methods are given in Table 1. As Amberlite IRA-904 does not have nitrogen containing groups [7], nitrogen estimated by *Kjeldhal* method would solely give the total nitrogen of the enzyme protein immobilized on Amberlite IRA-904. Immobilized protein estimated by *Kjeldhal* method and indirect method was almost same and no statistically significant difference was obtained (SE = 0.23 and 0.28, respectively; Table 1). In contrast, the protein estimated by elution method was only 68.9 % of that estimated by *Kjeldhal* method and differences between the mean values of protein estimated by these two methods was statistically significant (SE = 12.8 and 27.2). Therefore, indirect method was used in the rest of the experiments for the estimation of immobilized protein.

3.2 Activity of soluble and immobilized glucoamylase with different substrates

Activity yields of immobilized glucoamylase with different substrates are shown in Table 2. Activity yield with starch was only 2.2 % and it increased with decreasing molecular size of the substrate. Low activity of immobilized glucoamylase for starch could be due to the inaccessibility of the active site of the enzyme for bulky starch molecules. Average pore diameter of for Amberlite IRA-904 is 375 Å (range 210–1200 Å) [8]. Glucoamylase molecules with the molecular weight of 99,000 are about 150 Å [9] and hence can enter the pores of Amberlite IRA-904.

Amylopectin, a structural component of starch has a radius of gyration of 2000 Å [10]. Hence there is steric hindrance for amylopectin molecule to reach glucoamylase immobilized in the pores of Amberlite IRA-904. This limitation may permit the enzyme immobilized on the surface of Amberlite IRA-904 to hydrolyze starch molecules and hence the activity yield was low (2.2 %). On the other hand a reduction in steric hindrance by the use of small size substrates such as dextrinized starch and maltose had increased the molar concentration of substrates leading to enhanced activity yield of immobilized glucoamylase. Activity and relative activity of soluble glucoamylase with different substrates as shown in Table 2. Soluble glucoamylase has highest activity with 2.0 % (w/v) starch compared with the same concentration of dextrinized starch (DE 36) and maltose, which could be due to the affinity of the enzyme for high molecular weight substrate. *Gruesbeck* and *Rase* have reported that the percentage of hydrolysis of amylopectin, starch and maltose by soluble glucoamylase was 80, 71 and 40 %, respectively [10]. *Reilly* reported that the rate of hydrolysis of glucose pentamer was five times higher than that of maltose [11]. In our work the relative activity of glucoamylase with starch, dextrinized starch and maltose was 100, 52.2 and 37.5 %, respectively.

3.3 Effect of grinding

The immobilized glucoamylase present in the ground form showed 7.3, 1.6 and 1.1 fold higher activity than that of the bead form with starch, dextrinized starch and maltose, respectively (Table 2). These results again indicate the limitation caused by the macro reticular nature of Amberlite

Table 2. Activity and activity yield of Amberlite IRA-904 immobilized glucoamylase with different substrates (2 %, w/v) at pH 4.5 and 55 °C.

Substrate	Activity (U)			*Activity yield (%)		**Relative of soluble activity glucoamylase	@Effectiveness factor
	Soluble	Immobilized		Bead	Ground		
		Bead	Ground				
Starch	235.0	5.4	39.3	2.2	16.0	100.0	7.3
Dextrinized starch	125.0	28.0	45.6	22.3	35.7	52.2	1.6
Maltose	88.5	25.0	30.8	28.0	30.8	37.5	1.1

* Activity yield = Activity of immobilized enzyme/Activity of soluble enzyme 100
** Relative activity = Product formed with a particular substrate/Maximum product formed in that set of experiment 100
@ Effectiveness factor = Activity of ground form with a particular substrate/Activity of bead form with a particular substrate

$$*\text{Aktivity yield} = \frac{\text{Activity of immobilized enzyme}}{\text{Activity of soluble enzyme}} \times 100$$

$$**\text{Relative activity} = \frac{\text{Product formed with a paticular substrate}}{\text{Maximum product formed in that set of experiment}} \times 100$$

$$@\text{Effectiveness factor} = \frac{\text{Activity of ground form with a particular substrate}}{\text{Activity of bead form with a particular subsrate}} \times 100$$

IRA-904 on the accessibility of the substrate which could be presented as effectiveness factor [12]. Effectiveness factor for dextrinized starch did not show marked variation from maltose. This could be due to the action pattern of α-amylase (from *Bacillus licheniformis*) used for the preparation of dextrinized starch because here the major end products obtained are maltose, maltotriose and pentamaltose [13].

Acknowledgements

Authors thank the University of Jaffna and International Science Program for the financial assistance.

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Address of authors: *Santhirasegarampillai Balakumar, Vasanthy Arasaratnam* and *Kandiah Balasubramaniam.* Department of Biochemistry, Faculty of Medicine, University of Jaffna, Kokuvil (Sri Lanka). **Corresponding author:** Dr. (Miss) *V. Arasaratnam,* Department of Biochemistry, Faculty of Medicine, University of Jaffna, Kokuvil, Sri Lanka.

(Received: August 19, 1997).