J. Microbiol. Biotechnol. (2012), **22**(5), 628–636
http://dx.doi.org/10.4014/jmb.1108.08070
First published online February 7, 2012
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Stabilization of a Raw-Starch-Digesting Amylase by Multipoint Covalent Attachment on Glutaraldehyde-Activated Amberlite Beads

Nwagu, Tochukwu N.[1,2]*, Bartho N. Okolo[1], and Hideki Aoyagi[2]

[1]*Microbiology Department, Faculty of Biological Sciences, University of Nigeria, Nsukka, Nigeria*
[2]*Life Sciences and Bioengineering, Graduate School of Life and Environmental Sciences, University of Tsukuba, Japan*

Received: August 25, 2011 / Revised: January 3, 2012 / Accepted: January 4, 2012

Raw-starch-digesting enzyme (RSDA) was immobilized on Amberlite beads by conjugation of glutaraldehyde/ polyglutaraldehyde (PG)-activated beads or by crosslinking. The effect of immobilization on enzyme stability and catalytic efficiency was evaluated. Immobilization conditions greatly influenced the immobilization efficiency. Optimum pH values shifted from pH 5 to 6 for spontaneous crosslinking and sequential crosslinking, to pH 6–8 for RSDA covalently attached on polyglutaraldehyde-activated Amberlite beads, and to pH 7 for RSDA on glutaraldehyde-activated Amberlite. RSDA on glutaraldehyde-activated Amberlite beads had no loss of activity after 2 h storage at pH 9; enzyme on PG-activated beads lost 9%, whereas soluble enzyme lost 65% of its initial activity. Soluble enzyme lost 50% initial activity after 3 h incubation at 60°C, whereas glutaraldehyde-activated derivative lost only 7.7% initial activity. RSDA derivatives retained over 90% activity after 10 batch reuse at 40°C. The apparent K_m of the enzyme reduced from 0.35 mg/ml to 0.32 mg/ml for RSDA on glutaraldehyde-activated RSDA but increased to 0.42 mg/ml for the PG-activated RSDA derivative. Covalent immobilization on glutaraldehyde Amberlite beads was most stable and promises to address the instability and contamination issues that impede the industrial use of RSDAs. Moreover, the cheap, porous, and non-toxic nature of Amberlite, ease of immobilization, and high yield make it more interesting for the immobilization of this enzyme.

Keywords: Glutaraldehyde, Amberlite, immobilization, raw-starch-digesting amylase, stabilization

Amylases are employed in numerous industrial processes for the degradation of starch polymers or oligosaccharides to simple sugars. Major areas of amylase application include food, agrochemical, paper, textile, and detergent industries [36]. Recent advances in biotechnology have widened the field of application of amylases to medical, chemical, and bioanalytical determinations [7].

Amylases are produced from a wide range of microorganisms including bacteria, yeasts, and molds, with some of these organisms reported to secrete different types of extracellular amylases, which act synergistically in starch hydrolysis [27]. A minor percentage of these amylases are raw-starch-digesting amylases (RSDAs), which possess the starch-binding domain (SBD) and are therefore able to catalyze the hydrolysis of raw starch [14]. RSDAs only vary from other amylases in their special affinity and interaction with the microcrystalline structures of the raw starch molecule, through the SBD [34]. Limitations to the application of RSDA include low enzyme activity, poor stability, incomplete conversion, enzyme inhibition by glucose and maltose, problems of contamination due to application at low temperatures, and inability to degrade a wide range of starch, especially tuber starches such as potato starch [32].

Immobilization is an age-old method of biocatalyst stabilization, which additionally provides a multiphase system for easy recovery of products, reusability of enzyme, and continuous operation with more simplified, efficient, and cost-friendly processing [5]. Major techniques reported for immobilization of amylases include covalent binding, ionic and hydrophobic interaction, entrapment and aggregation. However, multipoint covalent attachment offers the most promise for total rigidification of enzyme molecule following immobilization [19]. Present on amylase surfaces are highly reactive lysine residues rarely involved in the active site of the enzyme [37]. These lysine groups are subject to modification by reaction with aldehyde, methyl, or carboxyl groups.

The choice of a carrier plays a significant role in biocatalyst immobilization. Amberlite is a macroporous strong-base

*Corresponding author
Phone: +2348036740060; Fax: +2348036740060;
E-mail: tochukwu.nwagu@unn.edu.ng

anionic resin with macroreticular crosslinked polystyrene matrix containing quaternary ammonium groups. Glutaraldehyde activation of Amberlite leads to the addition of aldehyde groups on the support surfaces, which subsequently react with lysine groups on the enzyme surface for covalent attachment and enzyme rigidification [20].

The isolation of *Aspergillus carbonarius* and production of copious amount of RSDA capable of saccharifying a wide range of starch sources were earlier reported [27]. A few reports on immobilization using Amberlite have been reported [1, 21, 29, 40]; however, none is available on immobilization of RSDA. These reports also showed that the property of the immobilized enzyme depended on the type of Amberlite bead used [35]. We report the stabilization of a RSDA from *A. carbonarius* using activated Amberlite IRA-910 beads. The Amberlite was either polyglutaraldehyde activated or simply activated with glutaraldehyde. Immobilization by ionic adsorption of the RSDA to Amberlite beads followed by crosslinking with glutaraldehyde was also carried out for comparative purposes.

Materials and Methods

Materials

Raw potato starch was prepared in our laboratory according to the method outlined by Okolo *et al*. [27]. Amberlite IRA-910 resin was used for the work. DNS was purchased from Lancaster, England. All other chemicals were of analytical grade and purchased from Wako Pure Chemicals, Japan.

Enzyme Production

RSDA was obtained from culture filtrate of *Aspergillus carbonarius* grown using the submerged fermentation (SmF) method. The pre-inoculum culture was prepared by inoculating two loopfuls of profuse growth into 500 ml Erlenmeyer flasks each containing 100 ml of sterile fermentation medium. The fermentation medium comprised (g/l) 20 raw corn starch, 2 yeast extract, 10 $(NH_4)_2HPO_4$, 1 NaCl, and 1 $MgSO_4 \cdot 7H_2O$ in deionized water. Cultures were incubated at 30°C with rotary shaking at 100 rpm for 24 h. After 24 h, 10 ml of the culture was used to inoculate a 500 ml flask containing 100 ml of the fermentation medium. The culture was cultivated for 96 h at 30°C, after which mycelial pellets were separated by filtration through sterile Whatman No. 1 filter paper. The resultant cell-free filtrate was purified through affinity chromatography using raw corn starch. The partially purified RSDA was crystallized using 80% ammonium sulfate as precipitant. The solution was stirred at 4°C for 4 h; excess ammonium sulfate was removed by decantation and the saturated solution kept for 36 h at −21°C. The crystals were suspended in citrate-phosphate buffer (pH 6.0) containing 0.1% starch solution at −21°C.

Preparation of Soluble Polyglutaraldehyde Solution

Soluble polyglutaraldehyde was prepared by the method of Tanriseven and Ölçer [38]. Glutaraldehyde solution [20 ml of 25% w/v, pH 10.5] was polymerized by the addition of 0.6 ml of 1 M NaOH solution at room temperature for 20 min. The reaction mixture was neutralized using an equal volume (0.6 ml) of HCl solution.

Immobilization on Inactivated Support

Prior to immobilization, Amberlite beads were properly washed with distilled water equilibrated with 0.2 M citrate-phosphate buffer, pH 6.0, and sucked dry, unless otherwise stated.

Adsorption of RSDA Followed by Crosslinking with Glutaraldehyde (Sequential Crosslinking)

Equilibrated Amberlite support (1 g) was suspended in 5 ml of 0.2 M citrate-phosphate buffer (pH 6) containing 10 mg of enzyme protein and stirred gently for 12 h at 25°C. The RSDA adsorbed onto Amberlite beads was recovered by filtration. This was followed by crosslinking using varying concentrations of glutaraldehyde at 30°C for 60 min. Crosslinked RSDA on Amberlite beads was filtered out of solution and thoroughly washed with 0.2 M phosphate buffer (pH 6.0). Immobilized RSDA was stored at 4°C.

Spontaneous Adsorption and Crosslinking of RSDA (Spontaneous Crosslinking)

To 1 g of equilibrated Amberlite support, 2.5 ml of 0.2 M citrate-phosphate buffer (pH 6.0) containing 360 U/ml RSDA and 2.5 ml of glutaraldehyde was added. This was followed by incubation at 30°C for 2 h. Immobilized amylase was thoroughly washed with 0.2 M citrate-phosphate buffer and stored at 4°C.

Activation of Carriers

Polyglutaraldehyde activation. This was done according to the method of Tanriseven and Ölçer [38]. Prior to activation, the Amberlite was washed with acetate buffer and sucked dry. Polyglutaraldehyde activation of the Amberlite was achieved by the reaction of 2 g of Amberlite beads with 10 ml of soluble polyglutaraldehyde in 0.2 M citrate-phosphate buffer (pH 8) for 15 min at 25°C using a magnetic stirrer. The activated carrier was washed repeatedly with distilled water for 10 min and reacted with glutaraldehyde [10 ml, 25% (w/v)] in acetate buffer (10 ml, 25 mM, pH 6) for 30 min at 30°C. The unreacted glutaraldehyde was removed from activated carrier by washing repeatedly with acetate buffer.

Glutaraldehyde activation. Glutaraldehyde activation was by treating the Amberlite beads with varying concentrations of glutaraldehyde (1.5−12%) for different durations (30 min to 6 h) at 30°C. Following the activation, carriers were thoroughly washed with distilled water and acetate buffer to remove excess glutaraldehyde.

RSDA Immobilization on Activated Amberlite Beads

Activated Amberlite support (1 g) was suspended in 5 ml of 0.2 M citrate-phosphate buffer (pH 6.0) containing 2 ml of RSDA, 360 U/ml and stirred gently for 24 h at 4°C. The Amberlite beads were recovered by filtration and thoroughly washed with distilled water and 0.2 M phosphate buffer (pH 6.0). Immobilized enzyme derivative was reduced with sodium borohydride (0.5 mg/ml, 4°C, 30 min) as earlier reported [33]. Immobilized RSDA was stored at 4°C.

Hereinafter RSDA immobilized on glutaraldehyde-activated support will be referred to as glutaraldehyde Amberlite-RSDA derivative (GAmb-RSDA), and RSDA immobilized on polyglutaraldehyde will be referred to as polyglutaraldehyde Amberlite-RSDA derivative (PGAmb-RSDA). Amb-RSDA-RET will signify spontaneous crosslinking, and AmbRSDA-CROSS signifies sequential crosslinking.

Optimization of Immobilization Method

Carrier activation and enzyme immobilization were performed at varying pH (5–7.5), and temperature (4°C, 10°C, and 25°C) values for different durations (6–36 h) while varying amylase concentrations (enzyme loading), followed by assaying of enzyme activity to determine optimal conditions for immobilization on each carrier.

Enzyme Activity and Protein

The raw-starch-digesting amylase activity was assayed using a reaction mixture containing 0.2 ml of 1% raw potato starch in 0.2 M citrate-phosphate buffer (pH 6.0), and 0.2 ml of enzyme solution, incubated at 40°C for 10 min in a bioshaker for homogeneity. Reducing sugars released after incubation were estimated by the DNS method of Miller [24]. One unit of amylase was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per minute under the assay conditions.

Determination of Protein Concentration

The amount of protein immobilized was estimated by subtracting the amount of protein determined in the supernatant after immobilization from the total amount of protein used for immobilization. The content of protein in solutions was determined by the Bradford method [2] using BSA as a standard and/or using the spectrophotometer at a wavelength of 280 nm.

Properties of the Enzyme Preparations

The optimum pH of the RSDA preparations was determined by incubating the RSDA preparations in 1% raw potato starch solution prepared in buffers of pH ranging from pH 3.0 to 9.0 at a temperature of 40°C. The pH stability of the RSDA of the soluble and immobilized RSDAs was studied by storing the enzyme in appropriate buffers of pH values ranging from pH 3.0 to pH 9.0 for 2 h at room temperature. Then, the residual amylase activity was estimated as described earlier.

The effect of temperature on the activity of the immobilized enzymes was determined by incubation at temperatures ranging from 30°C to 80°C for 20 min. Amylase activity was determined after incubation. Thermal stability of the enzyme preparations was determined by incubation in 0.2 M citrate-phosphate buffer (pH 6.0) at 60°C for 180 min. After incubation, the enzyme preparation was cooled and the residual activity assayed as described earlier. The effects of metal ions, surfactants, and inhibitors were examined by incubating immobilized RSDA preparations with these substances for 120 min. Thereafter, residual activity was determined.

To determine the operational stability of the immobilized enzyme preparations, after each batch reaction of starch hydrolysis (60 min) the beads were removed, washed thoroughly with distilled water, and stored at 4°C until the next use. The same process was repeated until the 10th cycle. The operational efficiency of the beads was calculated from the difference of the amount of product formed between the first and any of the subsequent batch reactions. Storage stability was tested after 30 days of keeping the immobilized enzyme preparations at 4°C. Effect of substrate concentration (0–1 mg/ml) was determined; K_m and V_{max} were calculated from Lineweaver–Burk plots.

All experiments were done in triplicates and the results represent mean values with less than 2.0% error.

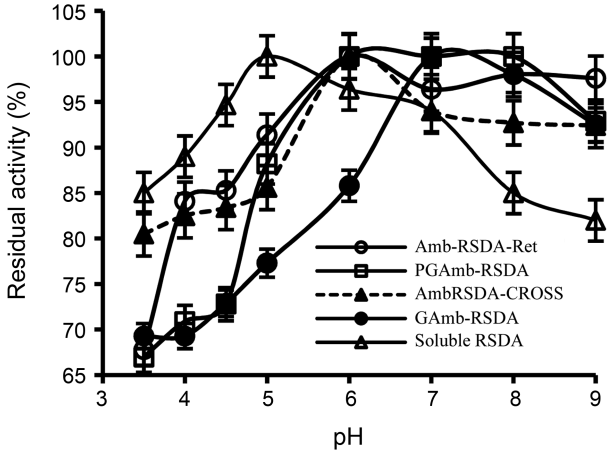

Fig. 1. Effect of reaction pH on activity of the enzyme.
Activity assays were performed using immobilized RSDA incubated at 40°C at different pHs with appropriate buffer solutions. The enzyme activity at optimum pH was considered as 100%.

RESULTS AND DISCUSSION

Optimization of RSDA Immobilization

Although a wide range of supports have been used for the immobilization of α-amylases and glucoamylases [15, 16], there is paucity of report on the immobilization of raw-starch-digesting amylases. RSDAs vary in structure from α-, β-, and gluco-amylases as a result of their starch binding domain (SBD) necessary for raw starch recognition and adsorption [14]. To minimize interference by other enzymes such as proteases, which are amongst the cocktail of enzymes released during fungal growth, partial purification was carried out. This was done in order to acquire a clearer picture as regards immobilization on this resin. Affinity chromatography using corn starch gave a purification yield of 83.3% and purification fold of 9. Further concentration and crystallization with ammonium sulfate increased the purification fold to 21 owing to a reduction in the total protein content (data not shown).

Glutaraldehyde Concentration and Duration of Incubation

Amberlite, an ionic resin, was used for the immobilization of RSDA through different methods: covalent modification on glutaraldehyde or polyglutaraldehyde (PG) activated beads, by sequential or spontaneous crosslinking. Process optimization greatly influenced the immobilization efficiency. The efficacies of the reactions were affected by the concentration of the glutaraldehyde, reaction time (bead activation/enzyme incubation), and reaction pH. A high concentration of glutaraldehyde (12%) was needed to activate Amberlite beads, whereas much lower concentrations of 2.5% and 3% were needed for spontaneous and sequential crosslinking, respectively (Table 1). The variation in glutaraldehyde concentration is a function of the role of the bifunctional agent in each process. The higher concentration

Table 1. Optimization of conditions for RSDA immobilization on Amberlite beads.

Conditions	Immobilization yield (%)			
	PGAmb-RSDA	AmbRSDA-CROSS	GAmb-RSDA	Amb-RSDA-RET
Glutaraldehyde concentration (%)				
2	-	69	50	50
2.5	-	74	59	60
3	-	75	61	53
5	-	70	64	52
9	-	-	66	-
12	-	-	77	-
Duration of glutaraldehyde activation/crosslinking (min)				
60	-	65	55	47
90	-	69	70	60
120	-	71	79	69
150	-	70	77	68
180	-	69	77	67
Incubation time (h)				
6	79	56	61	-
12	79	61	70	-
18	83	64	75	-
24	83	73	75	-
pH				
4.5	41	52	71	40
5	67	68	75	61
6	82	64	69	64
7	68	71	50	62
7.5	46	70	48	64

PGAmb-RSDA (polyglutaraldehyde-activated)
AmbRSDA-CROSS (sequential crosslinking)
GAmb-RSDA (glutaraldehyde-activated)
Amb-RSDA-RET (spontaneous crosslinking)

of glutaraldehyde needed for Amberlite activation is necessary to provide the optimum number of aldehyde groups on support surfaces for covalent attachment with lysine groups of the enzyme. However, utilization of a high glutaraldehyde concentration for crosslinking may lead to excessive rigidification of the enzyme structure and subsequent loss of activity. Moreover, for spontaneous crosslinking, a high concentration of glutaraldehyde may not only lead to excessive rigidification but may also cause conformational changes that may result in the damage of the enzyme active site. There was no variation in the length of time required for bead activation when the optimum glutaraldehyde concentration was used, as shown in Table 1; however, there was variation in the length of time required for enzyme incubation/immobilization. Maximum immobilization yields on glutaraldehyde and polyglutaraldehyde activated beads were achieved after 18 h incubation of enzyme with support matrix, while 24 h was needed for optimum adsorption of enzyme on non-activation beads prior to enzyme crosslinking (sequential). It is evident that covalent bond formation between enzyme active groups and the multiple aldehyde groups on Amberlite surfaces for glutaraldehyde- and PG-activated beads was more rapid compared with the rate of enzyme adsorption on non-activated beads through ionic and nonspecific charges. Prolonged incubation on activated support results in the formation of excessive multi-linkages, which cause steric hindrances and conformational changes in the enzyme, with resultant inaccessibility of the substrate to the active sites and loss of activity.

Effect of pH on Immobilization

Immobilization was best at a reaction pH of 5 for glutaraldehyde-activated beads, which corresponds with the work of Tripathi *et al*. [40] where pH of 5 gave the optimum immobilization yield for the conjugation of amylase on Amberlite beads. Platková *et al*. [29] reported an optimal pH of 5.7 for conjugation of fructosyltransferase on Amberlite beads. Enzyme immobilized on polyglutaraldehyde-activated beads had the best immobilization yield at pH 6, and for crosslinking, the optimum was at pH 7. A number of factors including enzyme pI, nature of linker, charges on support matrix, and enzyme surface determine the optimum pH for immobilization. Glutaraldehyde crosslinking is faster in neutral or basic conditions than in acidic conditions [38], which may explain the higher pH needed for optimum immobilization.

Immobilization Efficiency

Other properties of the immobilized enzyme are elucidated in Table 2. Immobilization yield was high and residual activities of immobilized enzyme exceeded 50% of fed-enzyme activities in all cases. RSDA on PG-activated beads gave the highest immobilization yield (92%), which is probably due to attachment of a higher number of aldehyde groups on Amberlite surfaces and their subsequent reaction with amino groups of the protein. Sharma *et al*. [35] reported that Amberlite IR-1204 with an immobilization yield of 69% was the best support for the immobilization of tannase from *Penicillium variable* compared with Amberlite XAD-7, silica, and DEAE-cellulose.

Effect of Immobilization on pH of the Enzyme

The pH activity profile of the RSDA was modified owing to immobilization. There was a shift in optimal pH from 5 of the soluble RSDA to 6 for the RSDA immobilized by spontaneous and sequential crosslinking. Apart from a slight shift of one unit to the basic side observed for RSDA on PG-activated bead, the optimum pH was also broadened to 6–8, whereas a positive shift of 2 units was observed for the derivative of glutaraldehyde-activated bead.

Shift of optimum pH to the alkaline side after immobilization has previously been reported [30, 42]. The pH of the

Table 2. Properties of the immobilization RSDA.

Bound RSDA type	Amylase activity (U/ml)	Total protein (mg)	Immobilization[a] Yield (%)	Expressed[b] activity (%)	% Activity after 30 days storage
Spontaneous crosslinking	240.4	8.0	ND[c]	68.0	101.0
PG-activated	244.4	8.9	92	73.2	109.3
Sequential crosslinking	213.7	7.1	76	64.0	97.8
Glutaraldehyde-activated	260.6	7.8	85	78.1	100.0
Soluble enzyme	360.0	10.0	ND[c]	ND[c]	70.0

[a]Immobilization yield (%) = (Amount of protein in the control suspension – Amount of protein in the supernatant of the immobilization suspension)/Amount of protein in the control suspension.
[b]Expressed activity (%) = Actual activity of the derivative/Expected activity considering the immobilized enzyme. Load in all experiments: 360 U/ml protein.
[c]ND = not determined.

immobilized enzyme is influenced by changes in the microenvironment of the carrier; inhibition of immobilized RSDA at lower pH may be due to a lower loading or possible changes in enzyme conformation due to unfavorable charge distribution on amino acid residues [31]. Moreover, a more alkaline environment may be required for optimal immobilized enzyme activity owing to the proton gradient generated by the support [26]. It could also be as a result of chemical groups from the glutaraldehyde molecules, which are more active at neutral or alkaline pH. The higher activity at alkaline pH implies that this enzyme may be invaluable for use in detergent, textile, and other industries where alkalophilic raw-starch-hydrolyzing amylases are of utmost importance. It is obvious that immobilization on polyglutaraldehyde-activated beads (PGAmb-RSDA) remarkably broadened the pH optima of the RSDA. Secondary interactions between the enzyme and the support based on their structures and charges affect intermolecular forces responsible for maintaining the conformation of the enzyme. The attached aldehydes may be responsible for the broadening of pH optimum towards the alkaline range. More interestingly, Fig. 2 shows that the pH stability of the RSDA increased after immobilization. All RSDA derivatives retained over 70% activity after storage at the acidic and alkaline pH of 3, 4, 8, and 9 for 120 min; however GAmb-RSDA was most stable with over 100% relative activity at pH 8 and 9. Although the free enzyme was more active at lower pH, immobilized RSDA was more stable at both acidic and alkaline pH. The prolonged storage in low pH may have led to the inactivation of the free enzyme owing to undesirable conformational changes [31]. The ability of the RSDA derivatives to show greater stability at pH 3, 4, 8, and 9 is an indication that immobilization reduced enzyme sensitivity to pH changes [13]. Lo and Ibrahim [21] reported that immobilization of lipase from *Pseudomonas* sp. AK resulted in improved pH stability.

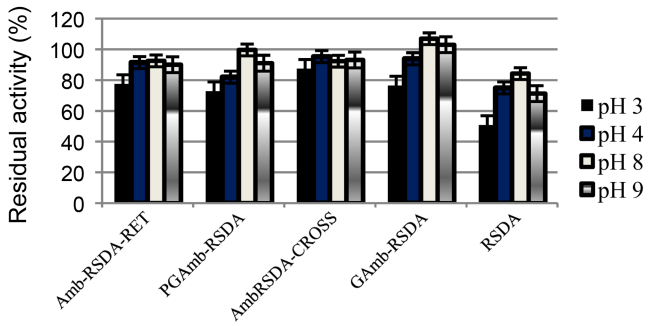

Fig. 2. Influence of pH on the stability of the RSDA.
Activity assays were performed using immobilized RSDA prepared with buffer of varying pHs and stored at 25°C for 120 min. Incubation was in a bioshaker for 20 min at 40°C. The enzyme activity at optimum pH was considered as 100%.

Storage Stability of the Immobilized Enzyme

Immobilized enzyme was stable when Amberlite-RSDA derivatives were stored semi-dry. After storage for 30 days at 4°C, it was observed that immobilization improved the storage stability of the RSDA derivatives to varying degrees. GAmb-RSDA, PGAmb-RSDA, and Amb-RSDA-RET maintained 100% of their activities (Table 2), and a slight decrease of 2.2% was observed for AmbRSDA-CROSS, whereas the soluble enzyme lost 30% of its initial activity. Enzymes easily lose their activities during storage or reuse owing to various environmental conditions. This was actually one of the problems identified with the native RSDA. It is imperative that storage and operational stabilities of an enzyme are evaluated prior to application in bioprocessing. These results showed that immobilization remarkably improved the storage stability of the RSDA and helped it to retain its activity for a longer period of time. Platkova *et al*. [29] reported that fructosyltransferase from *Aureobasidium pullulans* immobilized on Amberlite IRA-900 beads retained 98% of its initial activity after one month of storage.

Surfactants and Inhibitors on Immobilized Enzyme

During storage, transportation, or operation in biological reactions, biocatalysts are exposed to various chemicals from the storage containers, bioreactors, or even the reaction medium, which may lead to loss of activity or

Table 3. Effects of metal ions, surfactants, and inhibitors on activity of RSDA immobilized enzyme on Amberlite beads.

Agent	Conc	Activity (%)			
		RA[a]	PGA[b]	CXA[c]	GA[d]
EDTA	5 mM	98.0	98.1	85.8	92.8
Cholic acid	5 mM	100.0	100.2	100.4	93.1
Strontium chloride	5 mM	100.7	90.2	100.0	100.0
Sodium deoxycholate	5 mM	98.2	100.8	98.9	93.8
SDS	5 mM	100.6	100.0	88.6	100.0
Tween 80	0.5%	185.0	203.6	198.0	244.8
Triton X-100	0.5%	110.0	106.4	98.5	95.2

[a]RA = spontaneous crosslinking
[b]PGA = PG-activated beads
[c]CXA = sequential crosslinking
[d]GA = glutaraldehyde-activated

inactivation of the enzyme. The study of activating agents and enzyme inhibitors is therefore required for proper formulation of enzyme. Table 3 shows the effects of various metal surfactants and inhibitors on the activity of the immobilized enzyme. PGAmb-RSDA and Amb-RSDA-RET showed remarkable stabilization after exposure to 5 mM concentration of the inhibitors for 120 min at room temperature; over 90% activity was retained in all cases. Whereas 0.5% Tween 80 activated all immobilized derivatives, this was not the case when 0.5% Triton X-100 was used.

Effect of Immobilization on Temperature of the Enzyme

Immobilization did not alter the optimum temperature of the RSDA, as both the free and bound types had optimum temperature of 30°C; however, the range of activity of RSDA derivatives was remarkably broadened to a higher temperature range as shown in Fig. 3. In the case of PGAmb-RSDA, AmbRSDA-CROSS, and GAmb-RSDA, the optimal activity profile extended by 20°C, from 30–50°C in approximate values.

Tripathi *et al*. [40] also reported that the optimum temperature of mung beans α-amylase was not altered by immobilization on Amberlite beads. However, owing to changes in conformational flexibility, the bound enzyme may require a higher activation energy with which to reorganize its conformation to that necessary for catalysis [16, 36]. It is interesting that the immobilized RSDA showed increased activity at higher temperatures, as AmbRSDA-CROSS and GAmb-RSDA were most thermoactive with over 80% residual activity at 80°C. Surprisingly, PGAmb-RSDA and Amb-RSDA-RET lost over 20% of their activities at temperatures above 60°C. This could be due to the formation of multiple linkages on the enzyme at higher temperatures as a result of free aldehyde groups, leading to decrease in enzyme activity. Undersaturation of support could be responsible for the availability of free unstable aldehyde groups on support surfaces. Although RSDAs do not require gelatinization of starch and subsequent cooling of starch slurry prior to action, a little raise in reaction temperature would minimize the chances of contamination during bioprocessing and still conserve energy. It is worthy to note that previous works done in our laboratory on *A. carbonarius* RSDA showed the hydrolysis products as glucose and maltose with their concentrations dependent on the starch source [27]. This implies that when using this RSDA, the first stage of starch liquefaction will be by-passed. At the temperature of around 60°C, bound RSDAs would efficiently facilitate the saccharification of raw starch without prior gelatinization, saving time and cost with improved process efficiency.

Thermoinactivation kinetics of the enzyme revealed that the soluble RSDA lost half (50%) of its initial activity after

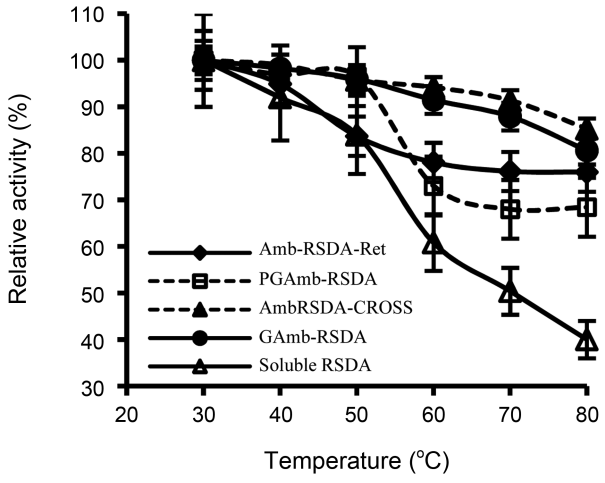

Fig. 3. Temperature activity profile of the RSDA.
Activity assays were performed using immobilized RSDA prepared with citrate-phosphate buffer (pH 6.0), incubated in a bioshaker for 20 min at varying temperatures. Optimum temperature was considered as 100%.

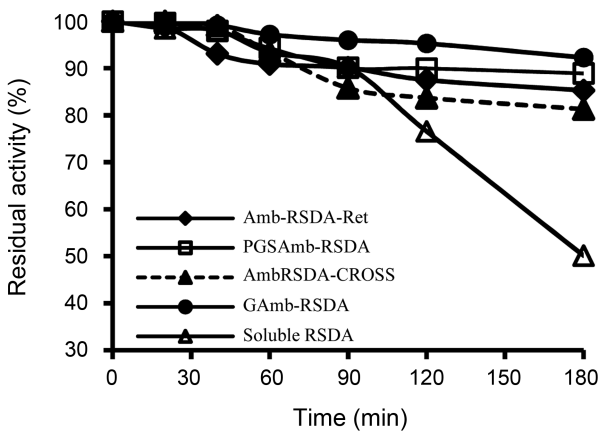

Fig. 4. Thermoinactivation kinetics of free and immobilized RSDAs on Amberlite beads at 60°C

storage at 60°C for 180 min (Fig. 4), while Amb-RSDA-RET lost only 14.7% activity, PGAmb-RSDA lost 11.1%, and AmbRSDA-CROSS lost 18.7%. GAmb-RSDA was the most thermostable and lost only 7.7% of its initial activity. Catalytic stability is an important parameter towards the industrial application of any biocatalyst. Unfortunately, this has been reported as one of the major limitations in the application of RSDAs [32]. Thermoinactivation kinetics of the RSDAs showed that the immobilized RSDA was significantly more thermostable than the free enzyme. This can be attributed to conformational changes in enzyme structure with a resultant rigidification of enzyme molecule [12].

Operational Stability of the Immobilized Enzyme

Immobilization ensured the reusability of the raw-starch-digesting amylase. Fig. 5 shows that immobilized RSDA derivatives were operationally stable and retained over 90% activity after 10 batch uses. Similar results were observed for β-galactosidase from peas immobilized onto Amberlite MB-150 beads [9].

Reusability of enzyme saves cost and affords ease of processing. Tripathi *et al*. [40] earlier reported that Amberlite-amylase showed a residual activity of 43% after 10 uses. Anita *et al*. [1] also reported that immobilized urease on Amberlite MB-1 retained 65% activity after 5 repeated uses. Enzyme loss during operation is as a result of denaturation or weakened bonds and ionic charges leading to enzyme leaching. It is obvious that covalent attachment between the bifunctional agent glutaraldehyde and enzyme active groups strengthened the quaternary structure of the enzyme, minimizing enzyme loss due to leakage or inactivation. Glutaraldehyde used for either pre-immobilization activation of the resin or post-immobilization crosslinking of the immobilized enzyme improves binding stability by rigidification of the enzyme structure through intermolecular forces between the matrix and enzyme [11].

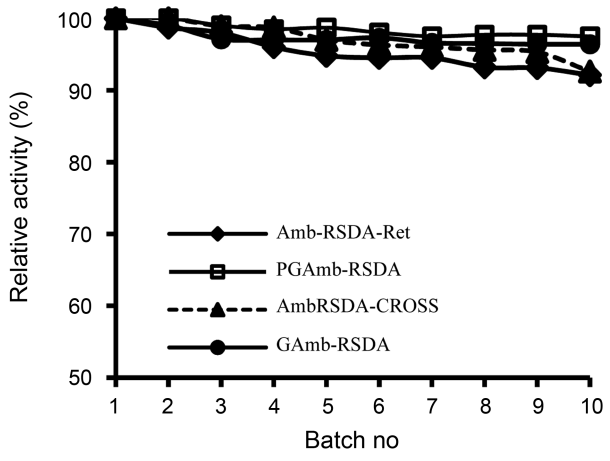

Fig. 5. Operational stability of the immobilized enzyme.

Kinetics Parameters of the Immobilized and Soluble RSDAs

The K_m (Michaelis–Menten constant) and V_{max} (maximum reaction rate) of the free and immobilized RSDAs were evaluated. Our results revealed that PGAmb-RSDA had an increased K_m value of 0.42 mg/ml compared with 0.35 mg/ml for the soluble RSDA (Table 4). Increase in K_m is an expression of loss of affinity of the enzyme for its substrate and commonly occurs after immobilization [8, 28]. This could be as a result of structural changes that occurred during immobilization, lower substrate accessibility to active site due to steric hindrances, or nature of the charges on support and enzyme molecules.

It is remarkable that a decrease in K_m was recorded for GAmb-RSDA and AmbRSDA-RET, with lowest value of 0.24 mg/ml recorded for AmbRSDA-CROSS. Reduction of K_m after immobilization does not often occur. However, a lower K_m was observed for α-amylase immobilized on cyclic carbonate bearing hybrid material [41] and α-amylase from *Aspergillus sclerotiorum* entrapped in calcium alginate beads [43]. Horse peroxidase immobilized on glutaraldehyde-activated polyaniline had a K_m value of 5.2 m mol l^{-1} while K_m for free enzyme was 9.8 m mol l^{-1} [10]. Mohapatra *et al*. [25] also reported the reduction in apparent K_m and increase in apparent V_{max} of sulfide oxidase from *Arthrobacter* species immobilized on glutaraldehyde-modified DEAE-cellulose. Entrapment of *Trichoderma reesi* endo-β-glucanase in alginate beads led to a slight reduction of K_m, from 1.31% of soluble enzyme to 1.02% [3]. A dramatic 7.5-fold decrease in K_m was reported by Chakrabarti and Storey [6] as a result of covalent immobilization of cellulase on hydrophilic polyurethane foam. Reduction in K_m can be attributed to a few factors, which include the complimentary charge properties of the carrier and the substrate, the ionic strength, the covalent modification of the amino group close to the enzyme active site, efficient mass transfer due to low enzyme load, and porosity of carrier [4, 5, 17]. Amberlite IRA-910 is a strongly basic anion-exchange resin with high porosity for the removal of large organic molecules. The charge–charge interactions between the positively charged substrate and the anion exchanger probably improved the enzyme's affinity for the substrate by modification of the enzyme

Table 4. Kinetic parameters of free and immobilized RSDA on Amberlite beads.

RSDA	K_m (10^{-1}) (mg/ml)	V_{max} (μmol/ml/min)$^{-1}$
Sequential crosslinking	2.6	17.7
RSDA + PG-activated	4.2	21.4
Spontaneous crosslinking	2.4	23.9
RSDA+Glutaraldehyde-activated	3.2	26.4
Soluble enzyme	3.5	23.8

conformation and molecular orientation. Kumari and Kayastha [18] reported that soybean α-amylase immobilized on Amberlite had an apparent K_m of 2.5 mg/ml, while on chitosan beads the apparent K_m was 4 mg/ml. Similar results were obtained by Tripathi *et al*. [40], indicating that the nature of the beads/carrier may have contributed towards improving the immobilized enzyme affinity for its substrate. Moreover, according to Tiller *et al*. [39], the coupling reagent plays a minor role in enzyme–substrate affinity compared with support material and density of protein. The high porosity of the carrier probably encouraged proper dispersal of enzyme molecules during immobilization, and also facilitated efficient transport of the large starch molecules during enzymatic hydrolysis minimizing steric hindrances or mass transfer limitations [23]. According to Mason and Weetal [22], no change in apparent K_m was observed when invertase was coupled to porous glass beads, suggesting the absence of diffusional barriers. The covalent attachment of aldehyde groups to amino groups (lysine groups) very close to the enzyme active site may also be responsible for the reduction in K_m. GAmb-RSDA exhibited the highest V_{max}, signifying a higher rate of reaction, and this is probably as a result of improved affinity of enzyme for its substrate. It is obvious that immobilization increased the catalytic efficiency of this derivative by producing a lower Michaelis–Menten constant and a higher rate of catalysis.

From the above results, RSDA covalently attached to glutaraldehyde-activated Amberlite beads (GAmb-RSDA) has the most promising results. GAmb-RSDA was active at high pH and temperature, and was also remarkably stabilized against extremes of pH and temperature. The enzyme had improved affinity for its substrate, which corresponds with the increase in V_{max} that was observed.

In conclusion, RSDA was successfully immobilized on Amberlite beads through various methods. RSDA immobilization on glutaraldehyde-activated beads gave the best overall result. The immobilized RSDA had a broader pH and temperature stability, showing the effectiveness of the support and the immobilization process. Immobilized enzyme showed storage and operational stability, a major factor in the use of a biocatalyst in industrial processes. Therefore, immobilization promises to address the problem of instability and aid in abating the issues of product contamination (by increased temperatures of operation) or enzyme inhibition by the product (by inaccessibility of substrate to substrate due to interactions with the matrix and multi-phase medium created by immobilization). The improved kinetic and stability features combined with the properties of the support (cheap, resistance to degradation, porosity) make this process a lot more appealing for industrial application. Immobilized amylase can find application in various industries including food, bioanalytical, pharmaceutical, and even biofuel, with improved yield and process efficiency.

Acknowledgments

The authors thank the University of Tsukuba, Japan for the Graduate Fellowship for T.N. The authors are grateful to Dr. Yoshida of the Graduate School of Life Science and Bioengineering, University of Tsukuba, Japan, for the assistance rendered throughout the course of this work.

References

1. Anita, A., C. A. Sastry, and M. A. Hashim. 1997. Immobilization of urease using Amberlite MB-1. *Bioprocess Eng.* **17:** 355–399.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72:** 248–254.
3. Busto, M. D., N. Ortega, and P. Mateoz. 2004. Characterization of microbial endo-β-glucanase immobilized in alginate beads. *Acta Biotechnol*. **18:** 189–200.
4. Cao, L. 2005. *Carrier-Bound Immobilized Enzymes: Principles, Applications and Design*. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim
5. Cao, L. 2005. Immobilized enzymes: Science or art? *Curr. Opin. Chem. Biol.* **9:** 217–226.
6. Chakrabarti, A. C. and K. B. Storey. 1988. Immobilization of cellulase using polyurethane foam. *Appl. Biochem. Biotechnol.* **19:** 189–207.
7. Cordeiro, C. A. M., M. L. L. Martins, and A. B. Luciano. 2002. Production and properties of α-amylase from thermophilic *Bacillus* sp. *Braz. J. Biotechnol.* **33:** 145–157.
8. Dey, G., V. Nagpal, and R. Banerjee. 2002. Immobilization of α-amylase produced from *Bacillus circulans* GRS 313. *Braz. Arch. Biol. Technol.* **46:** 167–176.
9. Dwevedi, A. and A. M. Kayastha. 2009. Stabilization of β-galactosidase (from Peas) by immobilization onto Amberlite MB-150 beads and its application in lactose hydrolysis. *J. Agric. Food Chem.* **57:** 682–688.
10. Fernandez, K. F., C. S. Lima, H. Pinho, and C. H. Collins. 2003. Immobilization of horseradish peroxidase on to polyaniline polymers. *Process Biochem.* **38:** 1379–1384
11. Fisher, J. 2008. Enzyme immobilization on polymeric resins: Amberlite and duolite strive to improve catalysis economics through reuse. *Genet. Eng. Biotechnol. News* **18:** 17.
12. Hasirci, N., S. Aksoy, and H. Tümtürk. 2006. Activation of poly (dimer acid-co-alkyl polyamine) particles for covalent immobilization of α-amylase. *React. Funct. Polym*. **66:** 1546–1551.
13. Ida, I. J., T. Matsuyama, and H. Yamamoto. 2000. Immobilization of glucoamylase on ceramic membrane surfaces modified with a new method of treatment using SPCP-CVD. *Biochem. Eng. J.* **5:** 179–186.
14. Janeček, S., B. Svensson, and E. A. MacGregor. 2003. Relation between domain evolution, specificity, and taxonomy of the α-amylase family members containing a C-terminal starch binding-domain. *Eur. J. Biochem*. **270:** 635–645.
15. Kahraman, M. V., G. Bayramoğlu, N. Kayaman-Apohan, and A. Güngör. 2007. UV-Curable methacrylated/fumaric acid modified epoxy as a potential support for enzyme immobilization. *React. Funct. Polym.* **67:** 1385–1392.

16. Kahraman, M. V., G. Bayramoğlu, N. Kayaman-Apohan, and A. Güngör. 2007. α-Amylase immobilization on functionalized glass beads by covalent attachment. *Food Chem*. **104:** 1385–1392.
17. Kotwal, S. M. and V. Shankar. 2009. Immobilized invertase. *Biotechnol. Adv*. **27:** 311–322.
18. Kumari, A. and A. Kayastha. 2011. Immobilization of soybean (*Glycine max*) α-amylase onto chitosan and Amberlite MB-150 beads: Optimization and characterization. *J. Mol. Catal. B Enzym.* **69:** 8–14.
19. Li, T., S. Li, N. Wang, and L. Tain. 2008. Immobilization and stabilization of pectinase by multipoint attachment onto an activated agar-gel support. *Food Chem.* **109:** 703–708.
20. Liu, X. D. and Y. Xu. 2008. A novel raw starch digesting α-amylase from a newly isolated *Bacillus* sp. YX-1: Purification and characterization. *Bioresour. Technol.* **99:** 4314–4320.
21. Lo, Y. S. and C. O. Ibrahim. 2005. Some characteristics of Amberlite XAD-7-adsorbed lipase from *Pseudomonas* sp. AK. *Malay. J. Microbiol.* **1:** 53–56.
22. Mason, R. D. and H. H. Weetal. 1972. Invertase covalently coupled to porous glass preparation and characterization. *Biotechnol. Bioeng.* **14:** 637–645.
23. Mateo, C., M. Palomo Jose, G. Fernández-Lorente, R. Fernandez-Lafuente, and J. M. Guisán. 2007. Improvement of enzyme activity, stability and selectivity *via* immobilization techniques. *Enzyme Microb. Technol.* **40:** 1451–1463.
24. Miller, G. L. 1959. Use of dinitro-salicylic acid reagent for determination of reducing sugars. *Anal. Chem*. **31:** 426–428.
25. Mohapatra, B. R., D. W. Gould, O. Dinardo, S. Papavinasam, D. W. Koren, and W. Revie. 2008. Effect of immobilization on kinetic and thermodynamic characteristics of sulfide oxidase from *Arthrobacter* species. *Prep. Biochem. Biotechnol*. **38:** 61–73.
26. Munjal, N. and S. K. Sawhney. 2003. Stability and properties of mushroom tyrosinase entrapped in alginate, polyacrylamide and gelatin gels. *Enzyme Microb. Technol*. **30:** 613–619.
27. Okolo, B. N., F. S. Ire, L. I. Ezeogu, C. U. Anyanwu, and F. J. C. Odibo. 2001. Purification and some properties of a novel raw starch-digesting amylase from *Aspergillus carbonarius*. *J. Sci. Food Agric.* **81:** 329–336.
28. Ortega, N., M. Perez-Mateos, M. C. Pilar, and M. Busto. 2009. Neutrase immobilization on alginate glutaraldehyde beads by covalent attachment. *J. Agric. Food Chem*. **57:** 109–115.
29. Platková, Z., M. Polakovič, M. Štefuca, M. Vandáková, and M. Antošová. 2006. Selection of carrier for immobilization of fructosyltransferase from *Aureobasidium pullulans*. *Chem. Pap.* **60:** 469–472.
30. Reshmi, R., G. Sanjay, and S. Sugunan. 2006. Enhanced activity and stability of α-amylase immobilized on alumina. *Catal. Commun*. **7:** 460–465.
31. Reshmi, R., G. Sanjay, and S. Sugunan. 2007. Immobilization of α-amylase on zirconia: A heterogeneous biocatalyst for starch hydrolysis. *Catal. Commun*. **8:** 393–399.
32. Robertson, G., D. Wang, C. Lee, K. Wasschal, M. Smith, and W. Orts. 2006. Native or raw starch digestion: A key step in energy efficient biorefining of grain. *J. Agric. Food Chem*. **54:** 353–365.
33. Rodrigues, D. S., W. Adriano, A. Mendesa, W. S. Adriano, L. Goncalves, and R. Giordano. 2008. Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods. *J. Mol. Catal. B Enzym.* **5:** 100–109.
34. Sarikaya, E., T. Higassa, M. Adachi, and B. Mikami. 2000. Comparison of degradation abilities of α- and β-amylases on raw starch granules. *Process Biochem.* **35:** 711–715.
35. Sharma, S., L. Agarwal, and R. K. Saxena. 2008. Purification, immobilization and characterization of tannase from *Penicillium variable*. *Bioresour. Technol*. **99:** 2544–2551.
36. Shewale, S. D. and A. B. Pandit. 2007. Hydrolysis of soluble starch using *Bacillus licheniformis* on superporous CELEBEADS. *Carbohydr. Res*. **342:** 997–1008.
37. Silva, C. J. M., F. Sousa, G. Gübitz, and A. Cavaco-Paulo. 2004. Chemical modifications on proteins using glutaraldehyde. *Food Technol. Biotechnol*. **42:** 51–56.
38. Tanriseven, A. and Z. Ölçer. 2008. A novel method for the immobilization of glucoamylase onto polyglutaraldehyde-activated gelatin. *Biochem. Eng. J*. **39:** 430–434.
39. Tiller, J. C., R. Rieseler, P. Berlin, and D. Klemm. 2002. Stabilization of activity of oxidoreductases by their immobilization onto special functionalized glass and novel aminocellulose film using different coupling agents. *Biomacromolecule.* **3:** 1021–1029.
40. Tripathi, P., A. Kumari, P. Rath, and A. Kayastha. 2007. Immobilization of amylase from mung beans (*Vigna radiata*) on Amberlite MB 150 and chitosan beads: A comparative study. *J. Mol. Catal. B Enzym.* **49:** 69–74.
41. Türünc, O., M. V. Kahraman, A. S. Akdemir, N. Kauahan-Apohan, and A. Güngör. 2009. Immobilization of α-amylase onto cyclic carbonate bearing hybrid material. *Food Chem*. **112:** 992–997.
42. Varavinit, S., N. Chaokasem, and S. Shobsngob. 2002. Immobilization of a thermostable alpha amylase. *Asia* **28:** 247–251.
43. Yagar, H., F. Ertan, and B. Balkan. 2008. Comparison of some properties of free and immobilized α-amylase by *Aspergillus sclerotiorum* in calcium alginate beads. *Prep. Biochem. Biotechnol.* **38:** 13–23.