Review Article

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Anti-enzyme antibodies: stabilization and therapeutic potential

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Abstract

Anti-enzyme antibodies can be utilized for various purposes. They have been used for single step immunoaffinity purification of enzymes and for their immobilization. Both polyclonal and monoclonal antibodies have been employed in the immobilization of enzymes. The resulting immobilized enzymes exhibit almost full activity and enhanced stability. Antibodies raised against the peptides corresponding to the labile region of enzymes can be used to prepare affinity supports that bind and selectively confer enhanced stability to them. Antibodies can facilitate folding and prevent aggregation of protein antigens. This chaperone-like antibody activity may prove to be a promising approach to the treatment of Alzheimer's and prion-related diseases. The antibodies have immense therapeutic potential and are being utilized to treat various diseases. This review aims at giving an overview on the stabilization of enzymes by antibodies and the therapeutic potential of the anti-enzyme antibodies.

Keywords: Enzymes; Antibodies; Stabilization; Therapeutic potential.

1. Introduction

Antibodies are considered the major tools of the trade to the immunochemists. They are able to recognize specifically every molecular structure (within certain dimensional limit) that man has isolated or synthesized, and they can distinguish between molecules as confusingly similar as two proteins differing by only one amino acid residue. Specific antibodies can be raised against any enzyme in suitable experimental animals and they can be utilized for various purposes. For example, anti-enzyme antibodies can be immobilized to a suitable matrix, and the affinity matrix thus generated may be used for the purification of the enzyme from the crude lysates in a single step (Silman and Katchalski, 1986; Ehle and Horn, 1990; Barbosa et al., 2015). Numerous immunoaffinity enzyme immobilization studies employ specific antibodies coupled to appropriate porous/non-porous solid supports in order to help facilitate ready mass transfer, heat transfer, etc. and offer good flow characteristics (Saleemuddin, 1999). A number of studies have been performed to show the stabilizing potential and chaperone-like activity of anti-enzyme antibodies. This review focuses on these applications of anti-enzyme antibodies and on their future potential as therapeutic agents.

2. Enzyme stabilization by antibodies

The most serious limitation in the long term and large scale applications of enzymes is their lability to various forms of inactivation. A number of strategies have been employed to improve the stability of enzymes (Wiseman, 1994). One of the most effective among these is immobilization on solid surfaces. Inspite of the remarkable achievements in improving the stability of several enzymes by immobilization, the

mechanisms involved are poorly understood, necessitating individual evaluation of the procedure for each enzyme by trial and error. Among the various strategies available for favourable orientation of enzymes (Turková, 1999), the use of monoclonal antienzyme antibodies is particularly promising (Solomon et al., 1987; Ruoff et al., 1989). Both polyclonal and monoclonal antibodies have been employed in the immobilization of enzymes and their relative merits and limitations examined (Saleemuddin, 1999) (Table 1). Many enzymes have been immobilized favourably with the help of antibodies (Younus, 2003). In each case, the immobilized enzyme preparation was more superior then the soluble one. Polyclonal antibody population is heterogenous. Formation of active site recognizing and hence inhibitory antibodies is quite likely if an animal is immunized with a native enzyme (Solomon et al., 1984; Cinader, 1967), although methods have been described for the generation of non-inhibitory antisera against several enzymes (Shami et al., 1991; Jafri et al., 1993; Feinstein et al., 1971; Ben-Yosef et al., 1975). Almost full activity by the enzyme complexed directly with antibody (Shami et al., 1989; Shami et al., 1991) or when bound to support matrix-coupled antibody has been observed (Stovickova et al., 1991). This is due to the fact that, the antibody molecule acts as a large spacer holding the enzyme at a distance from the support matrix thereby minimizing steric hindrance and facilitating remarkable freedom to act even on high molecular weight substrates (Solomon et al., 1986).

Enzymes immobilized on antibody supports usually exhibit high stability. The stability enhancement may arise out of crosslinking like effect caused by antibody binding on enzyme. Shami et al. (1989) argued that reduction in the free energy of the

antigen resulting from the binding of even a moderate affinity antibody may be sufficient to confer stability as free energy changes between the folded and the unfolded states of protein lie in the same range (Tanford, 1970). While the specificity of the antibody will be determined primarily by the individual side chain reactions, hydrogen bonding, and van der waals interactions, the affinity of the association and the reduction in free energy of the antigen are primarily due to hydrophobic interactions (Rees et al., 1988). Due to large size of the antibody, a single matrix bound antibody may not bind more than one molecule of the enzyme, lateral interactions with more than one antibody molecule may contribute significantly to the stability of the enzyme protein (Sadana and Madgula, 1993). In instances where soluble antibody is used for the enzyme immobilization, a single enzyme molecule may interact with more than one antibody molecules resulting in a high degree of stabilization (Shami et al., 1991) like an enzyme attached via multiple covalent or non-covalent linkages (Iqbal and Saleemuddin, 1983). While a correlation may exist between the thermal stability of proteins and its susceptibility to proteolysis (Daniel et al., 1982), the exact mechanism by which antibodies enhance stability against other forms of inactivation is still not clear. However, I postulate that since antibodies are formed against the exposed epitopes of a protein, many of which are loops on the surface which are loosely bound weak regions of the protein and hence are usually the first to unfold under any denaturing stress, therefore antibodies bind and prevent the unfolding of these regions and hence stabilize the protein to a good extent (Younus et al., 2001).

$F(ab)_2'$ (Jan et al., 2001) and Fab′ (Gupta et al., 2003) fragments of antibodies have also been used to successfully improve the stability of enzymes. While majority of

reports describe the insoluble enzyme preparations, complexing of antigenic enzyme with monomeric Fab′ results in the improvement of the stability of the former (Gupta et al., 2003). Such preparations have potential in enzyme therapy.

Studies using antibodies which bind to enzyme antigens at distinct and well-defined site(s), have led to a better understanding of the effects of enzyme-antibody interactions on enzyme behaviour (Solomon et al., 1996). By appropriate selection it has been possible to isolate those antibodies that are non-inhibitory and bind at strategic locations on the antigen molecule, leading to considerable stabilization of the enzyme conformation (Turková, 1999, Jafri and Saleemuddin, 1997, Solomon et al., 1987). The proposal of Ulbrich-Hofmann that enzymes contain "labile" region where the process of unfolding begins (Ulbrich-Hofmann et al., 1993) has been substantiated in a number of subsequent studies (Arnold et al., 1996; Mansfeld et al., 1999). It has been shown using pancreatic ribonuclease A (RNase A) as a model, that antibodies raised against the peptides corresponding to the labile region of enzyme can be used to prepare affinity supports that bind and selectively confer enhanced stability against thermal, pH and protease induced inactivation to the enzyme (Younus et al., 2001; Younus et al., 2002).

Although enzymes immobilized on antibody supports usually exhibit high operational and storage stability, binding of a recombinant phospholipase D from cabbage (PLD2) to antiPLD2 antibodies rendered the enzyme labile (Younus et al., 2004). It is likely that the antiPLD2 antibodies comprise populations of antibodies that labilize PLD2 by binding to epitopes of the enzymes crucial for its stability. We are not aware of any other reports on

anti-enzyme antibodies that enhance the lability of enzymes, although examples of enzymes turning labile to various forms of inactivation on immobilization are available (Sardar et al., 1997). While for most applications labilizing antibodies may be disadvantageous, they may find interesting applications in specific situations. The synthesis of high-amylose potato starch requires simultaneous inhibition of starch-branching enzymes A and B (SBE A and SBE B). Jobling et al., 2003 observed that an anti-SBE A single-domain antibody targeted to the plastids of transgenic potato plants deficient in SBE B could increase the amylose content of starch granules from about 20% to upto 74%. Labilizing antibodies may be more useful in restricting the role of enzyme without completely blocking their enzyme function.

3. Chaperone-like activity of antibodies

Protein folding *in vivo* or *in vitro* is often accompanied by formation of non-native conformations leading to protein aggregation. Many human disorders, such as Alzeimer's, Parkinson's, Huntington's and Creutzfeldt-Jakob's diseases are as a result of misfolding and aggregation of proteins (Dobson, 2003). Bacteria produce large quantities of recombinant proteins in rapid, often inexpensive, fermentation processes; however, the product of interest is frequently deposited in insoluble inactive aggregates or inclusion bodies. Therefore, strategies to prevent protein aggregation, as well as research for techniques favoring productive protein folding *in vivo* and to enhance renaturation *in vitro* of recombinant proteins are important tasks.

A number of reports indicate that antibodies can facilitate folding and prevent aggregation of protein antigens. Aggregation is caused by interactions between hydrophobic patches in partially folded polypeptide chains. A number of strategies causing the disruption of such hydrophobic patches reduce aggregation (Nieba et al., 1997). A second strategy involves the use of antibodies which preferentially bind hydrophobic patches away from the active site to protect the protein from intermolecular associations leading to aggregation (Katzav-Gozansky et al., 1996) (Fig. 1). The increase in renaturation yield in the presence of specific antibodies was first established for refolding of the enzyme acetyl cholinesterase after its thermal denaturation (Michaeli et al., 1969). Later antibody induced folding of a subunit fragment of bacterial tryptophan synthase (Blond and Goldberg, 1987) and S-protein fragment of RNase A (Carlson and Yarmush, 1992) was reported.

The influence of antibodies on folding is strictly antigen specific, nonspecific antibodies being incapable of influencing folding (Michaeli et al., 1969; Ermolenko et al., 2002). Antibodies which do not inhibit the biological activity of the antigen and bind with a similar affinity constant to their epitopes on the molecule exhibit a chaperone-like activity in the refolding of their antigen (Ermolenko et al., 2004). *In vitro* inhibition of aggregation of β-amyloid peptide that forms insoluble fibrils and plaques in the brain of Alzheimer patient by monoclonal antibodies against its N-terminal region was first reported by Solomon et al., 1996. The same monoclonal antibodies were effective in a partial disaggregation and solubilization of already formed fibrils (Solomon et al., 1997). They further suggested that preparing antibodies against the sites of an enzyme where

protein aggregation is initiated may lead to the understanding and prevention of protein aggregation. It seems likely that in most proteins, the termini are perhaps involved in initiation of oligomerization leading to aggregation (Liu et al., 2002). We have shown using RNase A as a model that non-inhibitory antibodies directed against specific epitopes of the enzymes involved in initiation of aggregation are effective in reducing the enzyme aggregation (Fig. 2) (Younus et al., 2006). A recent report showed the prevention of the seeding and spread of tau pathology in Alzeimer's disease with mouse monoclonal antibody against the N-terminal projection domain of Tau in transgenic mice (Dai et al., 2018). It has also been demonstrated that monoclonal antibodies against the native form of the cellular form of prion-protein (PrPc) inhibit prion propagation both *in vitro* (Peretz et al., 2001) and *in vivo* (White et al., 2003).

These studies reveal the chaperone-like activity of antibodies. In contrast to cellular chaperone proteins, the effect of antibodies is strictly specific and involves only the antigen protein. Chaperone-like antibody activity may be due to the stabilization of native antigen conformations or folding transition states, or screening of aggregating hydrophobic surfaces (Ermolenko et al., 2004). This activity of antibodies may prove to be a promising approach to the treatment of Alzheimer's and prion-related diseases. Antibody-assisted folding may enhance renaturation of recombinant proteins from inclusion bodies.

4. Therapeutic potential of antibodies

Therapeutic antibodies represent one of the fastest growing areas of the pharmaceutical industry. There are several monoclonal antibodies in clinical use and many more in clinical trials (Chames et al., 2009). Table 2 shows some of the monoclonal antibodies approved for therapeutic purpose. They are being used in the treatment of several major diseases including autoimmune, cardiovascular and infectious diseases, cancer and inflammation. Immunotherapy in the near future may become one of the promising methods of treatment of human disorders associated with enzyme aggregation. Immunotherapy targeting specifically the toxic conformational states of proteins will aid in developing potential strategies for therapeutic intervention in Alzeimer's disease and tauopathies (Bittar et al., 2018).

An important exciting future application of anti-enzyme antibodies in therapy is the protection of an enzyme against glycation-induced inactivation. Protection of Cu, Zn-superoxide dismutase against inactivation induced by glucose, ribose or fructose by antibodies (Jabeen and Saleemuddin, 2006) has been reported. Glycation or non-enzymatic glycosylation is a major *in vivo* source of reactive oxygen and carbonyl species. The cytotoxicity of glycation results from the inhibition of specific properties of proteins, cross-linkage, aggregation and protein precipitation. These modifications interfere with normal functioning of proteins, especially of those with long half-lives, and manifest themselves in a variety of progressive diseases of aging, including vascular disease, kidney disease, stiffness of joints and skin and Alzheimer's dementia, and the complications are exacerbated in uncontrolled diabetes. Several enzymes undergo glycation-induced loss of biological activity (Khan et al. 2014).

It is unlikely that the antibodies restrict glycation of an enzyme merely by masking the susceptible sites. Binding of the antibody may prevent unfolding of the enzyme resulting from initial glycation reaction and thereby restrict further glycation and inactivation (Fig. 3). Whereas potential of antibodies in the protection of enzymes and proteins against glycation induced inactivation *in vivo* certainly appears attractive, one can also envisage the possibility of using antibodies for increasing the effectiveness of the administered enzyme during enzyme therapy. The potential immunological complications of administration in human of polyclonal/monoclonal antibodies derived from animal sources can be minimized by a variety of strategies, including the use of single-chain antibodies and the "humanization" of the antibody (Sanz et al., 2005). As a result of these advances, antibodies are beginning to fulfill their potential as therapeutics.

Glycated albumin is a potential target of therapy in the treatment of diabetic complications (Cohen, 2003). For example, the db/db mouse is a genetic model of diabetes that develops renal lesions resembling those observed in human diabetic nephropathy (Cohen et al., 1996). The effect of treatment of diabetic db/db mice with a murine monoclonal antibody that specifically recognizes Amadori glucose adducts in rodents and human glycated albumin (Cohen and Hud, 1989) was a reduction in proteinuria and attenuated mesangial expansion (Cohen and Hud, 1994). The reno-protective response to antibody treatment was interpreted to reflect a reduction in circulating biologically active glycated epitopes, since it was accompanied by a significant decrease in the plasma glycated albumin concentration (Cohen, 2003).

Therefore, the strategy of blocking the formation or effects of glycated albumin by specific antibodies holds promise as a valuable therapeutic adjunct for the prevention and treatment of complications in human diabetes.

Recently, the nanobodies have evolved into versatile research and application tools for various biomedical and biotechnology applications. Nanobodies are a novel class of therapeutic proteins based on single-domain antibody fragments that contain the unique structural and functional properties of naturally-occurring heavy chain only antibodies. They are cloned and isolated single variable domains having full antigen binding capacity and are very stable. Their properties include nanoscale size, robust structure, stable and soluble behavior in aqueous solution, reversible refolding, high affinity and specificity for only one cognate target, superior cryptic cleft accessibility, and deep tissue penetration, as well as a sustainable source (Wang et al., 2016). They have good potential for targeting tumors, toxins, and microbes (Siontorou, 2013).

Anti-enzyme antibodies have some limitations that limit their widespread use as therapeutics. For example, the production cost is high, especially of monoclonal antibodies. Further, there is inadequate pharmacokinetics and tissue accessibility as well as impaired interactions with the immune system (Chames et al., 2009). These drawbacks necessitate further research.

5. Concluding remarks

In this review, various applications of anti-enzyme antibodies have been described. The ability of anti-enzyme antibodies for the immobilization and stabilization of the

enzyme is now well recognized. Hence, several industrial enzymes are being increasingly employed in the immobilized state. The chaperone-like activity of antibodies holds promise in the treatment of diseases related to protein misfolding and aggregation, such as Alzheimer's and prion related diseases. Perhaps the most exciting application area for antibodies is the inhibition of glycation-induced inactivation of proteins. Since glycated proteins are known to be involved in several human disorders, therefore, antibodies rendering protection against glycation of proteins will prove to be highly effective therapeutic tools in the treatment of several human diseases. Nanobodies represent the next-generation antibody-derived biologics with significant advances over conventional antibodies (Liu et al., 2018). They have diverse potential applications in biomedicine and biotechnology.

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Abbreviations

PLD2, recombinant phospholipase D from cabbage; PrPc, cellular form of prion-protein; RNase A, pancreatic ribonuclease A; SBE A, starch-branching enzyme A; SBE B, starch-branching enzyme B.

Conflict of Interest:

The author declares no conflict of interest.

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Figure Legends

Figure 1: Mechanism of chaperone-like activity of antibodies. In absence of antibodies (A). In presence of specific antibodies (B).

Figure 2: Reduction of enzyme aggregation by antibodies directed against specific epitopes of the enzyme involved in initiation of aggregation. Oligomers of RNase A formed by the swapping of the N-terminal α-helices of monomers and by the swapping of the C-terminal β-strands between the monomers (A). Lowering of RNase A aggregation by binding to antibodies directed against an epitope involved in initiation of aggregation (N-terminal region) (B).

Figure 3: A model to demonstrate the enzyme glycation reaction (A) and its protection by specific antibodies (B).

Table 1. Enzyme stabilized favourably with the help of antibodies.

Antibody	Enzymes	Reference
Polyclonal	β-Galactosidase, Gulonolactone oxidase, Transglutaminase, Chymotrypsin, Subtilisin, α-amylase, Glucoamylase, Trypsin, Urease, NAD glycohydrolase, Invertase, L-Hydantoinase	Saleemuddin, 1999
Monoclonal	Transglutaminase, Carboxy peptidase A, Lactate dehydrogenase, Glucose oxidase, Nitrate reductase, Horse radish peroxidase,	Saleemuddin, 1999
Glycosyl specific polyclonal	Invertase	Jafri and Saleemuddin, 1997
Epitope specific polyclonal	Ribonuclease A	Younus et al., 2001; Younus et al., 2002
$F(ab)_2$ fragment of polyclonal	Glucose oxidase	Jan et al., 2001
Fab fragment of polyclonal	Bromelain	Gupta et al., 2003

Table 2. Some monoclonal antibodies approved for therapeutic purpose

Generic name	Trade name	Antibody format	Antigen	Approved indication
Muromomab	Orthoclone	Murine, IgG2a	CD3	Allograft rejection in allogeneic renal transplantation
Abciximab	ReoPro	Chimeric, IgG1	GPIIb/IIIa r	Maintenance of coronary patency
Rituximab	Mabthera	Chimeric, IgG1	CD20	CD20-positive B-cell non-Hodgkin's lymphoma
Daclizumab	Zenapax	Humanized, IgG1	CD25 (Il2r)	Allograft rejection
Basiliximab	Simulect	Chimeric, IgG1	CD25 (Il2r)	Allograft rejection
Palivizumab	Synagis	Humanized, IgG1	Protein F	Respiratory syncytial virus (RSV inhibitor) in children
Infliximab	Remicade	Chimeric, IgG1	TNFα	Crohn's disease and rheumatoid arthritis
Trastuzumab	Herceptin	Humanized, IgG1	HER2/Neu	Metastatic breast cancer
Etanercept	Enbrel	huFcγ1/TNFr	TNFα and β	Autoimmune diseases such as ankylosing spondylitis
Gemtuzumab	Mylotarg	Humanized, IgG4	CD33	CD33-positive acute myeloid leukemia
Alemtuzumab	Mabcampath	Humanized, IgG1	CD52	B-cell chronic lymphocytic leukemia
Ibritomomab	Zevalin ^{90}Y	Mouse, IgG1	CD20	B-cell non-Hodgkin's lymphoma
Adalimumab	Trudexa	Human, IgG1 (PD)	TNFα	Crohn's disease and rheumatoid arthritis

Alefacept	Amevive	huFcγ1/LFA-3	CD2	Chronic plaque psoriasis
Omalizumab	Xolair	Humanized, IgG1	IgE	Treatment of asthma
Tositumomab	Bexxar ^{131}I	Murine, IgG2a	CD20	CD20-positive B-cell non-Hodgkin's lymphoma
Efalizumab	Raptiva	Humanized, IgG1	CD11a	Moderate to severe plaque psoriasis
Cetuximab	Erbitux	Chimeric, IgG1	EGFR	Metastatic colorectal and head and neck carcinoma
Bevacizumab	Avastin	Humanized, IgG1	VEGF-A	Metastatic colorectal and non-small cell lung carcinoma
Natalizumab	Tysabri	Humanized, IgG4	Integrin-α4	Multiple sclerosis
Ranibizumab	Lucentis	Humanized, IgG1	VEGF-A	Wet-type age-related macular degeneration
Panitumumab	Vectibis	Human, IgG2	EGFR	Metastatic colorectal carcinoma
Eculizumab	Soliris	Humanized, IgG2/4	C5	Paroxysmal nocturnal haemoglobinuria
Certolizumab	Cimzia	Humanized, IgG1	TNFα	Crohn's disease

EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

Reference: Chames et al., 2009

Figure 1

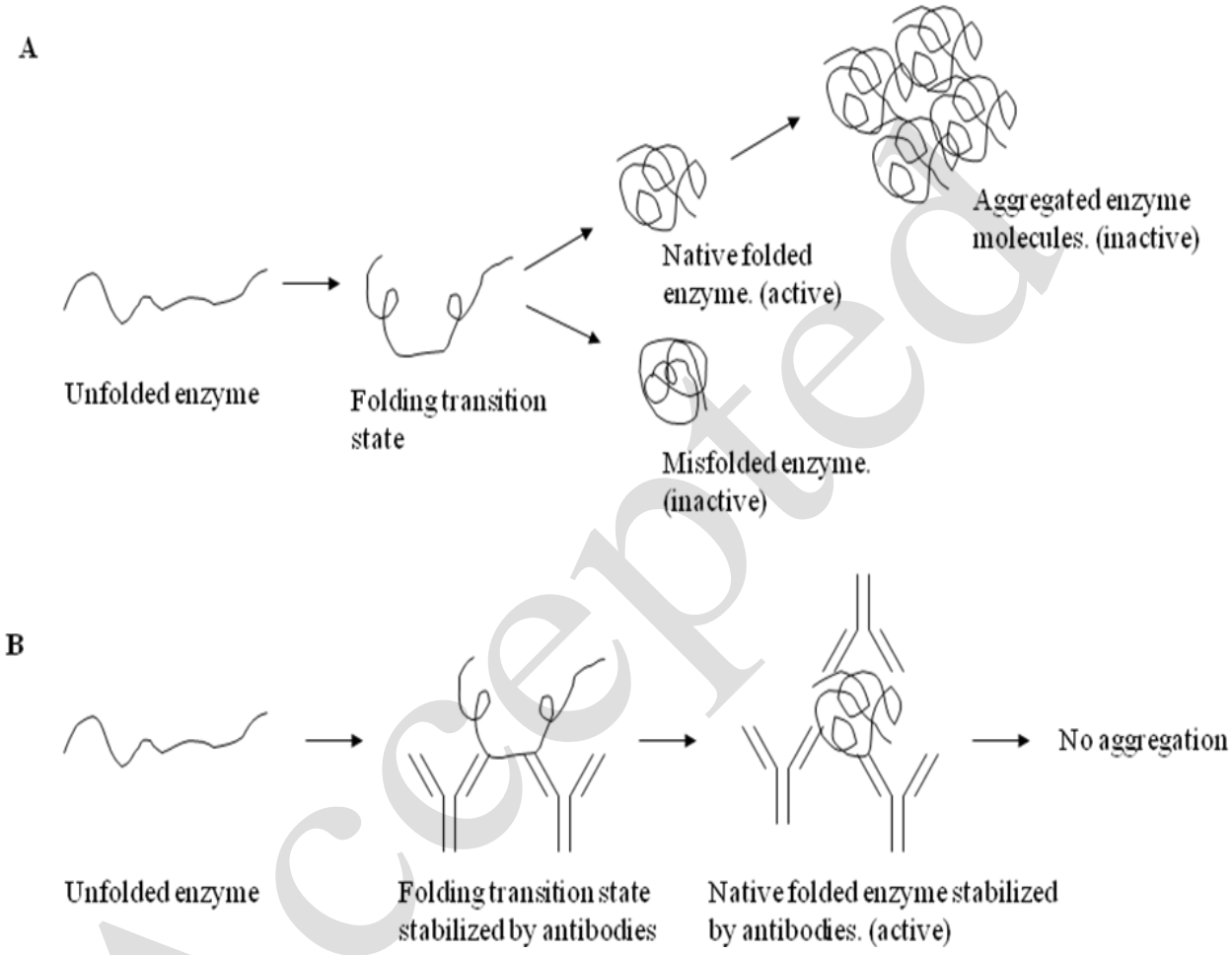

Figure 2

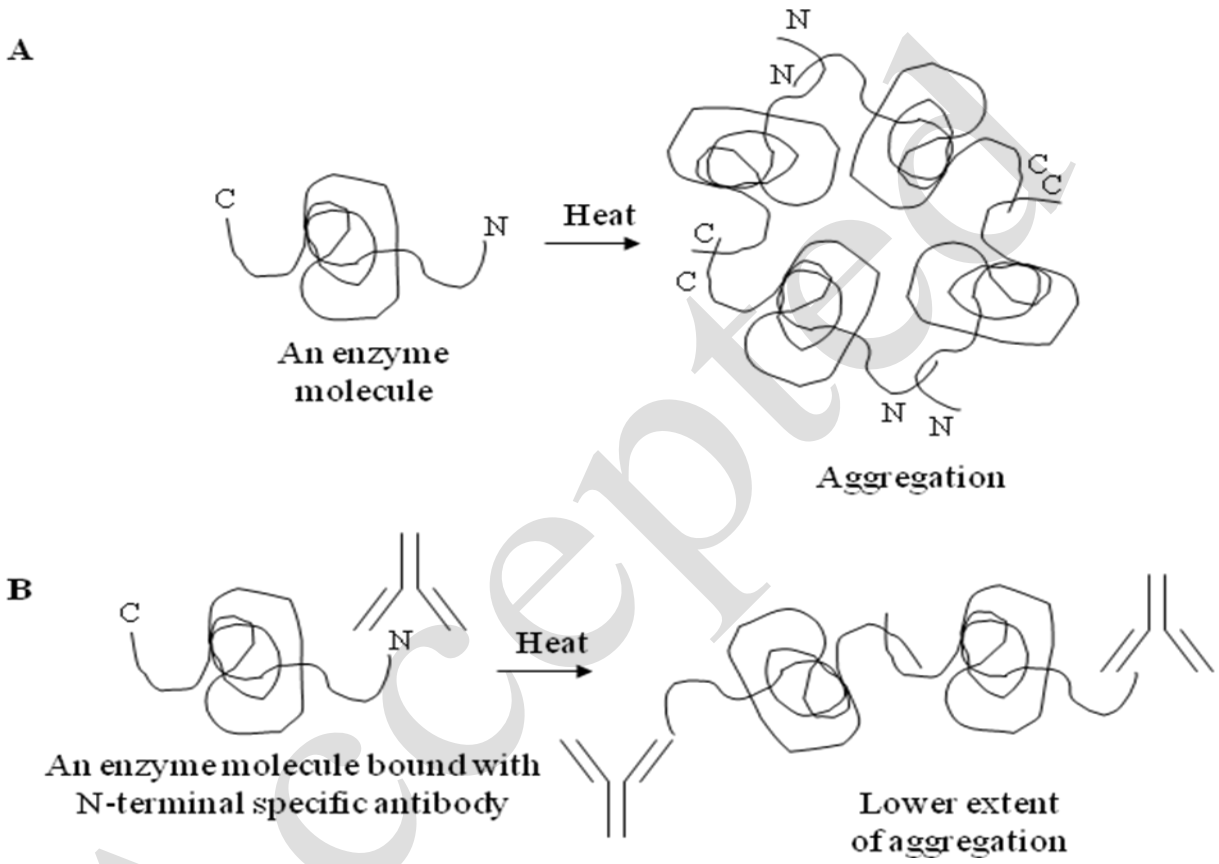

Figure 3