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Stabilisation of immunoassays and receptor assays

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Abstract

Stability of immunoassays and receptor assays depends on the integrity of antibodies and receptors as binding proteins for the analytes. In addition, coating conjugates, tracers, and if applicable, enzyme substrates have to be considered. In this study, temperature effects on monoclonal and recombinant antibodies for atrazine and on estrogen receptors were investigated in the presence and absence of stabilisers. Monoclonal antibodies for atrazine suffered only moderate activity losses after a 10-day incubation at 37°C which could be demonstrated by shifts of the calibration curves. Even more remarkable was the resistance of the analogous recombinant Fab fragments to temperatures up to 50°C. However, the estrogen receptor showed severe losses already after a 3-day exposure at 4°C. Various stabilisers were tested. In the case of antibodies, several commercial stabilisers prevented activity losses at higher temperatures. Improved stabilisation strategies are discussed. They are mainly seen in the selection of temperature-resistant recombinant antibodies and in the reduction of assay components.

Keywords: Stabilisation; Stabilisers; Immunoassays; Receptor assays; Antibodies; Receptors

1. Introduction

Immunoassays (IAs) are intensively used in the medical field, especially in clinical laboratories for therapeutic drug monitoring, control of hormone levels, viral diagnostics (hepatitis, HIV, etc.) as well as in biochemical and microbiological research. IAs are also applied to some extent in environmental analysis. IA technology is based on the biomolecular recognition of antigens or haptens by antibodies (abs), proteins that are produced by the body for the recognition of foreign material such as viruses or bacteria and their subsequent removal. The majority of present day IAs apply enzyme tracers to determine the ab binding sites that are not occupied by the analyte (competitive IAs) or binding sites that are occupied by the analyte (non-competitive IAs). Depending on the format, conjugates of analyte derivatives or abs with tracer enzymes are utilized. In addition coating conjugates have to be considered. Proteins are also involved in the case of fluorescence IAs, either as enzyme tracers generating fluorescent products or as fluorescent abs. Therefore stability problems in IA technology are primarily due to protein instabilities, followed by difficulties re-

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sulting from the decay of substrates and fluorescent labels.

Previous work of our group [1] was aimed at the stabilisation of IA components (abs, enzyme tracers, substrates) at elevated temperatures (30°C). Whereas lyophilised polyclonal abs could be stored for two weeks at 30°C with moderate losses, the activity of ab-coated plates decreased very fast under these conditions unless a post-coating step with polyvinyl alcohol (PVA), bovine serum albumin (BSA), fetal calf serum (FCS) or commercial stabilising solutions was applied. Commercial stabilising solutions were found to be suitable for stabilising enzyme tracers for several weeks at 30°C.

We have now extended our studies to a monoclonal (mab) and recombinant antibody (rab) for atrazine, and we investigated their response to higher temperatures in the presence and absence of stabilisers. In addition, we present data on estrogen receptor stability.

2. Theoretical background

Although it is easy to define stability, difficulties arise if we consider the question to which extent instability originating from limited storage, shelf life or half-life times can be tolerated. For the sake of simplicity, stability is defined here as $\mathrm{d}A/\mathrm{d}t = 0$, where A, activity; t, time. Therefore, IA stability can be improved by minimizing activity losses of individual IA components, resulting from ab, tracer or substrate decay.

The theoretical basis of protein stability has been layed down by Anfinsen [2] and reviewed in regular intervals, recently by Imoto [3], Lee and Vasmatzis [4], and Jaenicke [5]. Functional stability of proteins depends on the maintenance of integrity. However, proteins have been selected in evolution to be marginally stable. This means that the free-energy difference between the native state of a protein under physiological conditions and the inactive (unfolded) state of a typical 100-residue protein is ca. 10 kcal/mol [6]. This holds true not only for organisms living under mesophilic conditions, but also for hyperthermophiles growing optimally between 80 and 110°C [7]. Instability appears to provide protein flexibility as it is required for conformational changes during target binding and catalysis, trans-membrane transport in the nascent, partially unfolded form and easy degradation that renders unfolded proteins susceptible to degradation by proteases [6]. Therefore, protein instability is the result of biological evolution, but it appears to be counterproductive to many biotechnological applications.

Table 1 lists the major factors encountered in IA technology, which contribute to the destabilisation of proteins. Strategies to cope with protein instability can be broadly divided in two categories: avoidance and adaption.

The most frequent strategies applied to IA technology belong to the avoidance group. In this case suitable countermeasures such as storage at low temperature or the addition of stabilising reagents are taken to prevent instabilities that lead to inactivation and therefore result in weaker measuring signals and inferiour detection limits. The most critical factor is thermal denaturation leading to unfolding of proteins, inactivation, aggregation and precipitation. Frequently employed countermeasures include the addition of osmolytes such as polyols, sugars or amino acids. The stabilising effect has been related to their property of increasing the surface tension of water [8] and forcing out water layers from hydrophobic cavities in the interior of protein, followed by a decrease of material flexibility and fluctuational mobility of the water–protein matrix [9] as well as strengthening of the hydrophobic interaction of protein molecules [10]. Detailed studies on the stabilising effect of trehalose showed that it is the preferential binding to the native protein compared to the unfolded form which gives rise to the stabilisation. Therefore, the stabilising effect of this sugar could be fully accounted by the change in transfer free energy on unfolding [11].

Table 1
Factors contributing to instability and activity loss of native proteins; countermeasures frequently employed in immunoassay technology

Factor	Effect	Protective measures
High temperature	Thermal denaturation (unfolding of proteins, aggregation, and precipitation)	Polyols (glycerol, sorbitol, inositol, erythrol) and other osmolytes (e.g., sucrose, L-proline, L-serine, γ-aminobutyric acid, sarcosine, taurine, glycine, betaine) PVP Dextrane sulfate Heat-resistant chaperones Chemical cross-linking (intra- or intermolecular) Modification of protein structure by genetic engineering (substitution of individual amino acid residues, stabilisation by multiple S–S bonds, glycosylation)
Freezing	Denaturation by removal of solvent. Concentration of proteases in non-frozen part during slow freezing	Stabilisation by glycerol, BSA
Lyophilisation	Freezing- and drying-induced denaturation (aggregation)	Cryoprotection by polyethylene glycol, polyalcohols, amino acids
Drying	Drying-induced inactivation	Stabilisation by sugars (lactose, trehalose) or mannitol
Dilution	Destabilisation by loss of protein–protein interaction	Weakly interacting cosolvents. Addition of protective proteins (BSA, OVA, betain)
Proteolytic degradation	Fragmentation of proteins	Glycerol Short heat treatment of antisera and ascites fluids Proteinase inhibitors Ab purification, e.g., by affinity chromatography Modification of protein structure by genetic engineering (fusion proteins)
Chemical changes	Oxidation; Deamidation	Addition of SH-reagents (mercaptoethanol, dithiothreitol)
Plastic surfaces	Unspecific adsorption of dissolved proteins to hydrophobic surfaces, aggregation and denaturation	Detergents (e.g., Tween 20, Triton X-100) Block polymers (polypropylene glycol/polyethylene glycol) Use of glass vials

Problems are also frequently encountered during lyophilisation where freezing- and drying-induced denaturation take place. Studies by Gibson [12] on additives based on multiple electrostatic interactions have been carried out with enzymes. Favourable results were obtained with soluble polyelectrolytes that gave protein–polyelectrolyte complexes, followed by the addition of solutions of polyalcohols or other compounds containing multiple hydroxyl groups. Dehydration of the resulting solution by vacuum evaporation, freeze-drying or forced air convection produced a dry film or powder of stabilised protein.

Further factors resulting in loss of protein activities are listed in Table 1. Principally, these recommendations apply to most abs, enzymes or even receptors although specific features that are due to the enormous structural and functional diversity of proteins should be taken into account.

Adaptive strategies appear to be the most promising ones because they employ mutative changes where specific amino acid residues are genetically altered to provide lasting and higher stabilities without changing protein activity.

3. Experimental

3.1. Stabilisers

Six different stabilisers were applied for preserving ab activities. BSA (1%), PVA (5%) and lactitol (β-galactoside sorbitol, 10%) were obtained from Sigma. The CSIRO stabiliser was kindly provided by Dr. J. Skerritt (Canberra, Australia), formulations of the vinylpyrrolidone copolymers Gafquat® HS-100 and Gafquat® 755N (ISP Global Technologies, Frechen, Germany) by Dr. T. Gibson (Leeds, UK).

3.2. Antibodies

The mab K4E7 for atrazine has been developed in our group by Dr. T. Giersch [13]. It was produced in serum-free medium and purified by protein A chromatography and then lyophilised without stabilisers. Stock solutions were prepared by dissolving 2 mg in 200 μl 0.9% NaCl.

Recombinant Fabs (rFabs) for atrazine were derived from the cell line producing the mab K4E7 according to Ref. [14]. In brief, the variable heavy and light chain encoding genes of K4E7 were selectively amplified by PCR and sequentially cloned into the expression vector pASK85. This vector has been particularly designed for the production of Fab fragments since it bears the complementing constant domain gene cassettes C_H1 and C_L of a murine antibody as an inherent feature [15]. Following transformation of *Escherichia coli* and induc-

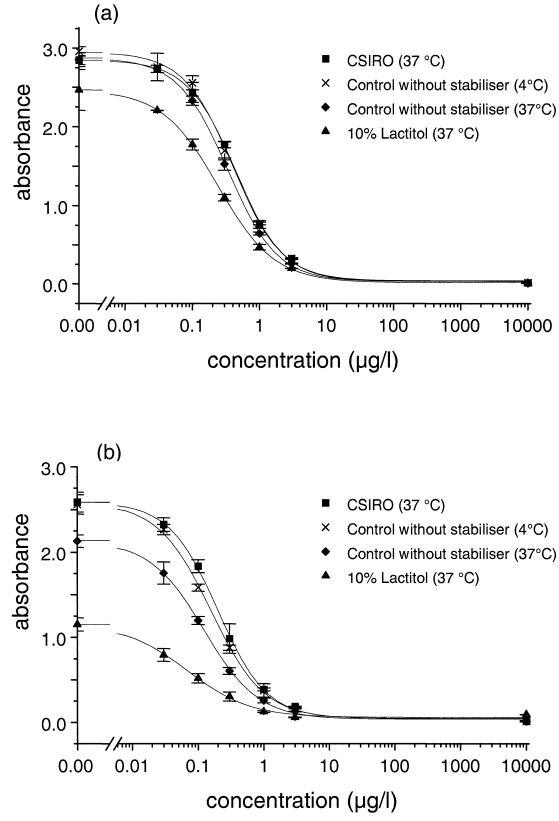

Fig. 1. Calibration curves for atrazine obtained with an EIA based on the mab K4E7 in the presence of different stabilisers (a) after a 3-day exposure of the mab at 37°C, (b) after a 10-day exposure under the same condition. Error bars indicate standard deviations obtained from four values.

tion of expression, the antibody light chain and the corresponding heavy chain moiety F_d are secreted into the periplasm of the transformants. Protein folding, association of functional Fab heterodimers and formation of interchain disulfide bonds is accomplished in this bacterial compartment. Expressed Fab fragments were purified from cell lysates by affinity chromatography employing immobilized Zn^{2+}. This strategy takes advantage by a histidine hexamer extension at the C-terminus of the heavy chain moiety exhibiting an appropriate affinity for Zn^{2+} ions. rFab stock solutions contained ca. 0.1 μg/ml.

3.3. Receptors

The recombinant human estrogen receptors α (ER) were obtained from two different sources. One was purchased from PanVera (Madison, WI, USA); the stock solution contained 1.4 μg/ml. The other ER was expressed according to McDonnell et al. [16] in our laboratory. The stock solution was 0.15 μg/ml. In brief, *Saccharomyces cerevisiae* was transformed with an expression plasmid (a gift from Dr. D.P. McDonnell) containing the gene for the human ER. After induction with Cu^{++}, the receptor was expressed as an ubiquitin fusion protein. Then the cell walls were removed by enzymatic digestion and the cells were lysed by hypotonic shock to liberate the receptor. The receptor was partially purified by affinity chromatography using heparin agarose. The recombinant receptor was undegraded, soluble and biologically active.

3.4. Enzyme immunoassay (EIA)

Competitive EIAs with the atrazine mab K4E7 were carried out as before [13]. Flat-bottomed polystyrene microtiter plates (Greiner) were precoated overnight with 300 μl of goat antimouse serum (Sigma; 5 μg/ml of carbonate buffer: $NaHCO_3/Na_2CO_3$ 50 mM, pH 9.6) at 4°C. All further reactions were carried out at room temperature. Following three repeated

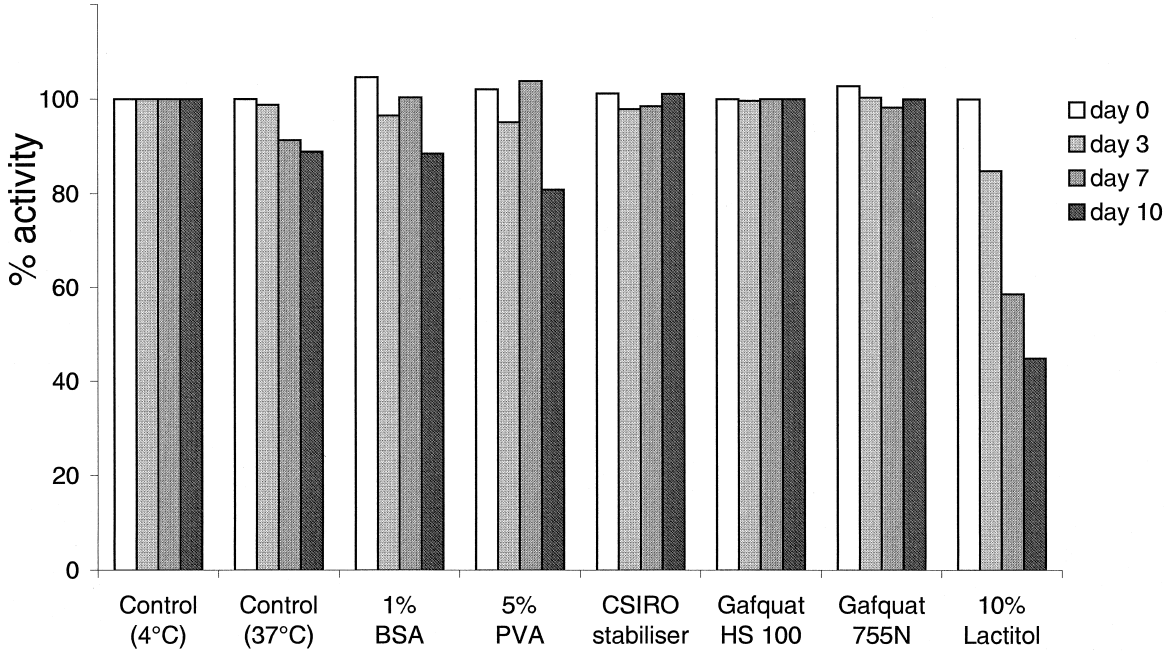

Fig. 2. Time course of the EIA signal at zero dose after incubating the mab K4E7 at 37°C in the presence or absence of stabilisers indicated in the legend. The absorbances of the 4°C controls (without stabiliser) were set to 100% activity.

washes with PBS–Tween 20 (4 mM PBS, pH 7.2, supplemented with 0.05% Tween 20), 300 μl mab K4E7 were prediluted 1:10 in stabiliser and kept at elevated temperature as described in the individual experiments. The abs were brought to a final dilution of 1:40,000 with PBS and filled into individual wells. Unbound mabs were removed by rinsing three times with PBS–Tween 20 after 2 h incubation. A total of 200 μl atrazine standards or PBS were added together with 50-μl enzyme tracer conjugate (a POD-atrazine derivative, diluted 1:40,000 in PBS) and incubated for 1 h. After an additional wash step, 200 μl peroxidase substrate were pipetted to each well. The substrate consisted of two parts of phosphate buffer (140 mM; pH 5.0) containing 3 mM of urea peroxide and one part of 1.2 mM tetramethylbenzidine (TMB) in 8 mM phosphoric acid supplemented with 10% dimethyl sulfoxide and 12 mg/l penicillin G to control bacterial growth. Substrate reaction was terminated after a defined time period (5–10 min) with 50 μl H_2SO_4 (4 M). The absorbance was measured at 450 nm with a Multiscan ELISA reader (ICN-Flow, Meckenheim).

Competitive EIAs with the rFab for atrazine were performed accordingly except for the following changes: The rFab prediluted 1:2 in stabiliser and kept at 50°C for the time period indicated in the experiment was brought to a final dilution of 1:10 by PBS. Individual wells were filled with 250 μl affinity purified Fab instead of 300 μl mab K4E7.

3.5. Enzyme-linked receptor assay (ELRA)

Receptor preparations were diluted 1:10 in 80 mM PBS (pH 7.6) and the stabilising solutions BSA (1%) and Gafquat® 755N. Incubations were carried out for 0 h, 48 h and 66 h at 4°C.

ELRAs were performed according to Ref. [17]. The final receptor dilutions were brought by PBS to 1:1000 in the case of the PanVera ER and 1:100 in the case of the own production. 96-microwell plates were coated overnight with 200 μl of an estradiol–BSA conjugate (Sigma, 1 μg/ml of carbonate buffer: $NaHCO_3$/Na_2CO_3 50 mM, pH 9.6) at 4°C. All subsequent reactions were carried out at room temperature; solutions were prepared in PBS. After washing plates three times with PBS–Tween 20 (4 mM PBS, pH 7.2, supplemented with 0.05% Tween 20) and blocking for 1 h (3% BSA; 2.7% Boehringer Mannheim blocking reagent), estradiol (Sigma) solutions of defined concentrations (50 μl, 0 μg/l–1000 μg/l) were added together with the ER and incubated for 1 h. After a further washing step, a biotinylated mouse anti-estrogen receptor-ab (Dako Diagnostica, Hamburg) was added and incubated for 1 h. Then an additional wash-step followed and 200 μl of a streptavidin–POD–biotin enhancement system (10 ng/ml streptavidin, Sigma; 2.5

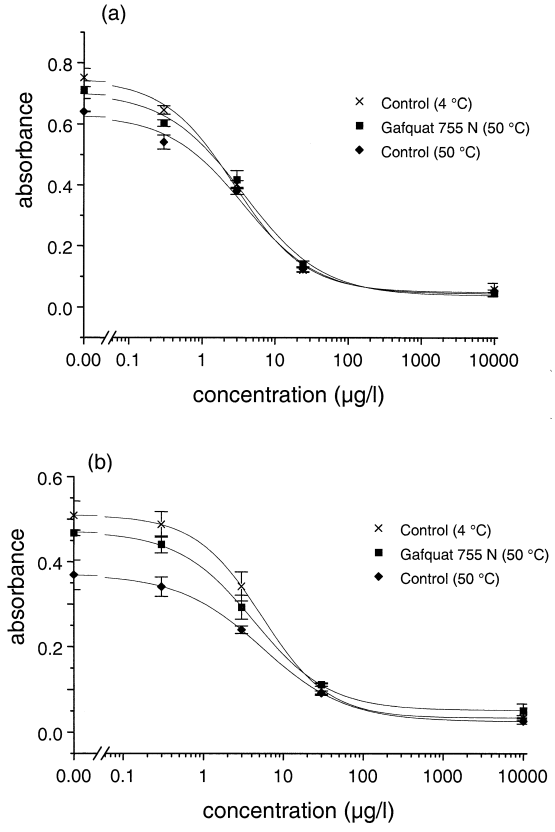

Fig. 3. Calibration curves for atrazine obtained with an EIA based on the rFab K47H in the presence of different stabilisers (a) after a 3-day exposure of the rFab at 50°C, (b) after a 5-day exposure under the same conditions. Error bars indicate standard deviations obtained from four values.

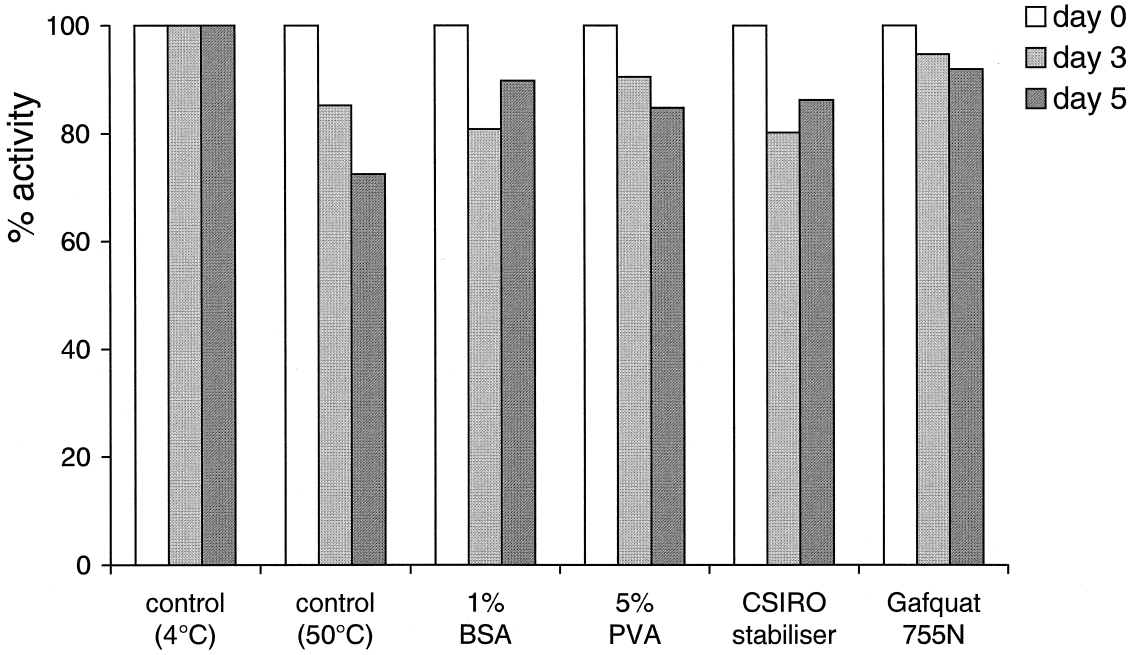

Fig. 4. Time course of the EIA signal at zero dose after incubating the rFab K47H at 50°C in the presence or absence of stabilisers indicated in the legend. The absorbances of the 4°C controls (without stabiliser) were set to 100%.

ng/ml POD–biotin, Sigma) were pipetted into each well and incubated for 1 h. After washing the plate peroxidase substrate (Sigma) was added (200 μl). The substrate consisted of two parts: one part of phosphate buffer (140 mM; pH 5.0) containing 3 mM of urea peroxide and one part of 1.2 mM TMB in 8 mM phosphoric acid supplemented with 10% dimethyl sulfoxide. The substrate reaction was terminated after 20 min with 50 μl H_2SO_4 (2 M) and absorbances (450 nm) were measured with an ELISA reader (EAR400, SLT-Tecan, Salzburg, Austria).

3.6. Data analysis

Data analysis for EIAs and ELRAs was performed with the aid of a commercial ELISA software package (SLT). The absorbances were normalised by the transformation to $\%B/B_0$ according to

$$\%B/B_0 = \frac{A - A_{\text{excess}}}{A_0 - A_{\text{excess}}} \times 100$$

where A is the absorbance, A_0 the absorbance at zero dose analyte, and A_{excess} the absorbance at an excess of analyte.

4. Results

Experiments on ab stability were initially performed under temperature regimes that are expected during transportation in tropical and subtropical climates. For this purpose, stock solutions of an atrazine mab were diluted 1:10 at the start of the experiment with stabiliser and kept for the indicated time period at 37°C. Then the mabs were brought with PBS to their final volume and the EIAs were carried out at room temperature with enzyme tracers and substrates stored under standard conditions at 4°C in the refrigerator. Therefore, any absorption shifts in the EIA can be attributed to temperature effects on the mab.

Fig. 1 shows representative calibration curves for atrazine obtained with the mab K4E7 kept

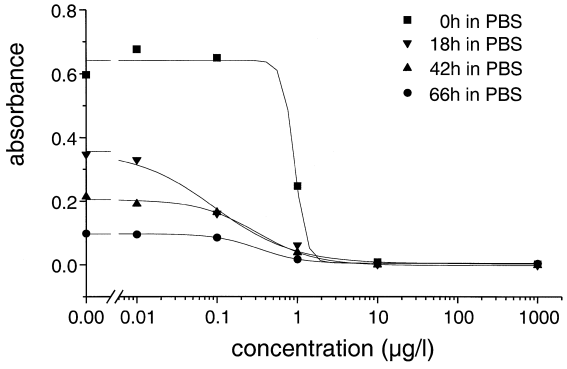

Fig. 5. Calibration curves for β-estradiol obtained with the ELRA based on the human ER after incubating the ER at 4°C for different time periods. Means of two values are given.

for 3 days (Fig. 1a) and 10 days (Fig. 1b) at 37°C in the presence or absence of the CSIRO stabiliser or lactitol, respectively. In order to obtain more detailed informations on the time course of temperature-dependent ab inactivation, the absorbancies of the zero controls (absence of atrazine) were compared on a %-basis (A_0 of the 4°C control was set to 100%) and were plotted against the exposure time (Fig. 2). It is obvious that except for lactitol there are only moderate losses of this mab during a 10-day exposure at 37°C compared to the controls at 4°C. The efficient stabilisers were Gafquat® HS100, Gafquat® 755N, and the CSIRO stabiliser.

Preliminary tests with the corresponding rFab that has been derived from the mab K4E7 showed that there was negligible inactivation at 37°C (Dankwardt, unpublished). Therefore, subsequent experiments with this recombinant ab were performed at 50°C to challenge rFab stabilities. Representative calibration curves for atrazine obtained with this rFab after 3-day exposure and 5-day exposure are depicted in Fig. 3a and b, respectively. The absorbancies of the zero controls, plotted on a %-basis against the exposure time, are shown by Fig. 4. It is obvious that even after five days at 50°C there are only moderate losses of the rFab which can be compensated to some extent by the stabilisers applied in this experiment, most efficiently by Gafquat® 755N.

The situation is entirely different with receptors. Stability tests were performed with the human estrogen receptor α (ER). Similar to the EIAs, competitive tests (ELRAs) were carried out which are based in this case on the competition for free ER binding sites between the analyte and an estradiol–BSA conjugate. Because of the low stability of the ER that can only be stored without losses at −80°C or below, the temperature exposures were carried out at 4°C in the presence or absence of 1% BSA and Gafquat® 755N. Fig. 5 presents representative calibration curves for 17 β-estradiol, the most potent natural estrogen. The time course of temperature dependent inactivation is depicted by Fig. 6 for two different ER preparations. It is obvious that for both, the commercial and the

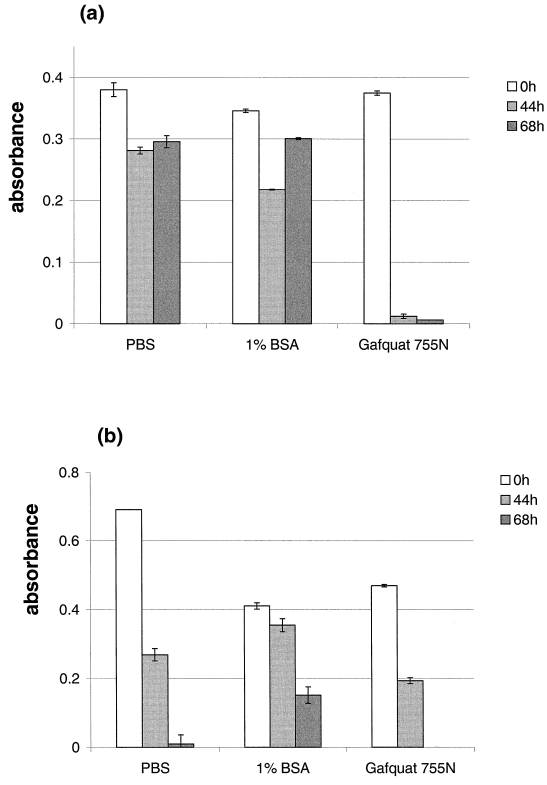

Fig. 6. Time course of the ELRA signal at zero dose after incubating the ER (a, laboratory preparation, b, commercial ER, PanVera) at 4°C in the presence or absence of BSA and Gafquat® 755N.

non-commercial ER there is a rapid inactivation in the presence of Gafquat® 755N, the most efficient stabiliser for the mabs. Receptor inactivation (PBS) occurs considerably slower in the case of the non-commercial ER; for both preparations, addition of 1% BSA appears to provide acceptable stabilisation, although it was found to slow down the assay in the case of the commercial receptor (Fig. 6b). Within the same time of colour development the sample (0 h) with BSA reaches about 60% of the intensity of the setup without BSA.

5. Discussion

Due to their protein nature, the functional stability of abs and receptors depends on the integrity of their three-dimensional structure. Unless specific precautions are taken, they are subject to denaturation ranging from slight conformational changes to the loss of solubility and massive irreversible aggregation in the case of in vitro preparations. Under natural conditions abs are protected within the specific environment of blood and lympha, especially in the presence of other proteins such as albumins. However, there are intrinsic differences in the stability of different abs. This is reflected by different half lives, which is approximately 23 days for human IgGs and only 6 days for IgMs under physiological conditions. Receptors are often much less stable. Their intensive turn-over within cells appears to originate from the necessity of providing free binding sites to trigger signal transduction.

The most frequent causes for in vitro ab and receptor instabilities are thermal denaturation, including activity losses during freeze–thaw cycles and drying. The most commonly used provision is the addition of stabilisers. Sugars, polyols and proteins have been applied in the past for thermal stabilisation and for preventing freeze/thaw-induced aggregations. Formulations with mannitol, trehalose, lactalbumin plus sucrose or glycine were used for stabilisation during freeze-drying and prolonged storage at elevated temperature.

In this paper, comparative studies on thermal effects on mabs, rFabs and ER were performed in the presence and absence of stabilisers.

Affinity purified mabs raised in serum-free medium showed only moderate losses after a 10-day exposure at 37°C whereas 50°C leads to a fast inactivation (not shown). In contrast to the mab, its corresponding rFab that has been derived from this mab [14] and exhibits the same pattern of cross reactivities (although at lower affinities) exhibits a remarkable stability at 50°C. It is not clear, yet, whether it is the absence of the Fc part or other factors that may lead to the increased stability of the rFab. Since the mab was applied in an affinity-purified form, it seems to be unlikely that the lower stability at elevated temperatures is due to the presence of proteinases.

The experiments with both, mab and rFab show that the presence of stabilisers such as the cationic conditioning copolymers Gafquat® 755N and HS-100, the CSIRO stabiliser, or BSA can abolish ab inactivations at elevated temperatures. This pattern fits well into the formulations summarised in Table 1.

Due to the enormous diversity of proteins, it is difficult to predict the effect on other abs or proteins. This becomes evident by the stabilisation experiments with the ER, an instable protein whose life time is restricted even at 4°C. The comparison of a commercial and a non-commercial ER with the same binding properties showed different decays at 4°C and the complete ineffectiveness of one of the stabilisers that proved to be most effective for ab stabilisation.

As it has already been pointed out by Skerritt [18], concern about reagent stability is the major difference in emphasis in IA development between research and commercial laboratories. Stabilisation is usually not covered by text books on IAs, but treated intensively by the patent literature. Therefore, informations are not readily available, apart from the difficulties resulting

from the different responses of individual abs or receptors to elevated temperatures. Therefore different proteins have to be tested individually.

Effective measures to cope with ab instability include elevated temperature treatments in screening programmes for both, mabs and rFabs. Furthermore, it is most important for the future to reduce the number of assay components. This has already been realised in the development of direct immunosensors that only use coating conjugate and do not require enzyme tracers. An even more rigorous step would be the omission of coating conjugates; this is possible in homogeneous test formats that only utilise the binding reaction between abs and the analyte followed by a separation of free and bound abs by means of LC methods.

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References

[1] A. Dankwardt, J. Müller, B. Hock, Anal. Chim. Acta 362 (1998) 35.
[2] C.B. Anfinsen, Biochem. J. 128 (1972) 737.
[3] T. Imoto, Cell Mol. Life Sci. 53 (1997) 215.
[4] B. Lee, G. Vasmatzis, Curr. Opin. Biotechnol. 8 (1997) 423.
[5] R. Jaenicke, Biochemistry (Moscow) 63 (1998) 312.
[6] L. Stryer, Biochemistry, 4th edn., Freeman, New York, 1995, p. 432.
[7] R. Jaenicke, FEMS Microbiol. Rev. 18 (1996) 215.
[8] T.Y. Lin, S.N. Timasheff, Protein Sci. 5 (1996) 372.
[9] S.P. Rozhkov, Biofizika 36 (1991) 571.
[10] K. Gekko, J. Biochem. (Tokyo) 90 (1981) 1633.
[11] G. Xie, S.N. Timasheff, Biophys. Chem. 64 (1997) 25.
[12] T. Gibson, Dev. Biol. Stand. 87 (1996) 207.
[13] T. Giersch, J. Agric. Food Chem. 41 (1993) 1006.
[14] K. Kramer, B. Hock, Immunoassays for Residue Analysis, in: R.C. Beier, L.H. Stanker (Eds.), ACS Symposium Ser. 621, Washington, DC, 1996, p. 471.
[15] A. Skerra, Gene 151 (1994) 131.
[16] D.P. McDonnell, Z. Nawaz, C. Densmore, N.L. Weigel, T.A. Pham, J.H. Clark, B.W. O'Malley, Mol. Biol. 39 (1991) 291.
[17] M. Seifert, H. Haindl, B. Hock, Adv. Exp. Med. Biol. 44 (1998) 113.
[18] J.H. Skerritt, New Frontiers in Agrochemical Immunoassay, in: D.A. Kurtz, J.H. Skerritt, L. Stanker (Eds.), AOAC International, Arlington, USA, 1995, p. 13.