Biochimica et Biophysica Acta, 615 (1980) 392–401

BBA 69121

THE EFFECT OF PROTEIN CONCENTRATION ON THE ACTIVITY OF β-FRUCTOSIDASE FROM RADISH SEEDLINGS

LOÏC FAYE and CLAUDE LAMBERT

Laboratoire de Photobiologie, Centre de Biochimie et Physiologie Cellulaires, Faculté des Sciences de Rouen, 76130 Mont Saint Aignan (France)

(Received March 18th, 1980)

Key words: β-Fructosidase; Activation; (Radish)

Summary

Radish β-fructosidase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) is inactived by diluting the enzyme solution and the activity can be restored by addition of bovine serum albumin or other proteins.

The use of detergent, high molar salt solutions or silicone-coated tubes showed that decrease of specific activity upon dilution is not linked to adsorption of the enzyme on to glass walls.

Albumin neither protects the enzyme from denaturation by heat nor changes its stability during conservation at room temperature. The action of added proteins is not due to removal of an inhibitor from the enzyme solutions.

Some polyanions or polycations have the same effect as albumin, but dialysis or chromatography show that they do not act by reassociation of inactive products formed by dilution of the active enzyme.

A molecular weight heterogeneity is observed in the enzyme population when chromatography is performed without albumin. This suggests that inactive forms, formed upon dilution, differ slightly in their molecular conformation from the active forms obtained at high protein concentration.

Introduction

Many enzymes have been found to be inactivated when diluted below a given protein concentration. Generally this phenomenon can be reversed by addition of exogenous proteins such as albumin. The published data dealing with this subject are numerous but generally descriptive. However, this enzyme behaviour is of great importance not only for evaluation of specific activity but also in physiological studies on comparison of enzymatic activities from extracts

which differ in their protein concentration.

Enzyme levels in higher plants are changed by a variety of environmental factor perturbations. But most of these studies are not performed with highly purified enzymes and poor information is provided concerning protein concentration, which may be an interfering agent in enzyme assay. In such conditions, an experimental stimulus inducing changes in the protein concentration of the extract, can produce artificial values for activity of enzymes, the specific activity of which is affected by exogenous protein concentration.

Radish β-fructosidase (β-D-fructofuranoside fructohydrolase EC 3.2.1.26) has been shown to be a glycoprotein [1] and this property is used to develop its purification (Faye, L. and Berjonneau, C., unpublished data). From purified samples of β-fructosidase we have observed that this enzyme is easily inactivated in dilute solution. This phenomenon is reversible upon the addition of proteins: the β-fructosidase activity of a solution with a low protein concentration may be far higher when the protein concentration is raised by addition of exogenous proteins.

In this study, we attempt to explain the reversible inactivation of this hydrolytic enzyme in dilute solution.

Materials and Methods

Reagents. Ovalbumin and bovine serum albumin fraction V were from Sigma. Albumin was further purified by chromatography on a Con A-Sepharose (Pharmacia) column according to Aspberg and Porath [2].

Rabbit immunoglobulins were isolated from rabbit sera according to Harboe and Ingild [3] and freeze-dried for storage.

Casein and all other chemicals were from Merck.

Enzyme source and assay. β-Fructosidase was isolated from light-grown radish seedlings irradiated for 72 h with standard far-red light source [1]. The purified preparation used here (Faye, L. and Berjonneau, C., unpublished data) was obtained by Sephadex G-25 (Pharmacia) chromatography followed by $(NH_4)_2SO_4$ precipitation, Con A-Sepharose (Pharmacia), Ultrogel ACA 34 and ACA 54 (Industrie Biologique Française) chromatography. The resulting preparation was not completely contaminant free but showed a high specific activity: 150 μkatal/mg as compared to 2 μkatal/mg for a crude extract. These specific activities were measured in the presence of albumin (for details see Results).

β-Fructosidase activity was measured as described previously [1] with minor modifications, if any, as detailed in Results. The reducing sugars formed were measured according to Nelson [4]. All dilutions, additions of reagents or preservations were made directly from a stock solution of purified enzyme in the test tubes used for assay.

Protein determination. Protein concentration was determined by the method of Lowry et al. [5] using albumin as standard. For the analysis of column effluents, absorbance at 280 nm was measured.

Gel filtrations. A column (0.9 × 50 cm) of Ultrogel ACA 54 was equilibrated with 85 mM citrate phosphate buffer, pH 6.5 containing 0.5 M NaCl in the presence or absence of (see Results) 0.3 mg/ml albumin. Enzyme sample (0.5 ml) was diluted and eluted with the same buffer at a flow rate of 3.1 ml/h and collected as 0.8-ml fractions.

Results

Effect of addition of albumin, ovalbumin, casein or rabbit immunoglobulins on β-fructosidase activity. Increasing amounts of the four proteins tested were added to a standard reaction mixture containing the same low β-fructosidase concentration (0.04 mg/ml). The enzyme activity observed with increasing concentrations of these four proteins is shown in Fig. 1. When the activity in absence of protein is expressed as 100, an increase to a final value of 250 is always observed when the protein concentration is raised to 100 μg/ml.

The maximal activity observed is the same with the four proteins used; the final protein concentration to reach the upper plateaus of the concentration/activity curves is 0.3 mg/ml for albumin or ovalbumin, and 0.6 mg/ml for casein or immunoglobulin.

These tests were performed at an enzyme concentration high enough to easily measure β-fructosidase activity without addition of exogenous proteins. However, activation was most marked at lower β-fructosidase concentration: when highly diluted extracts are used the enzyme is completely inactivated and reversibly reactivated by addition of protein (data not shown).

β-Fructosidase dilution and specific activity. Fig. 2 shows the evolution of specific activity when enzyme is diluted in buffer alone (curve A), or in buffer

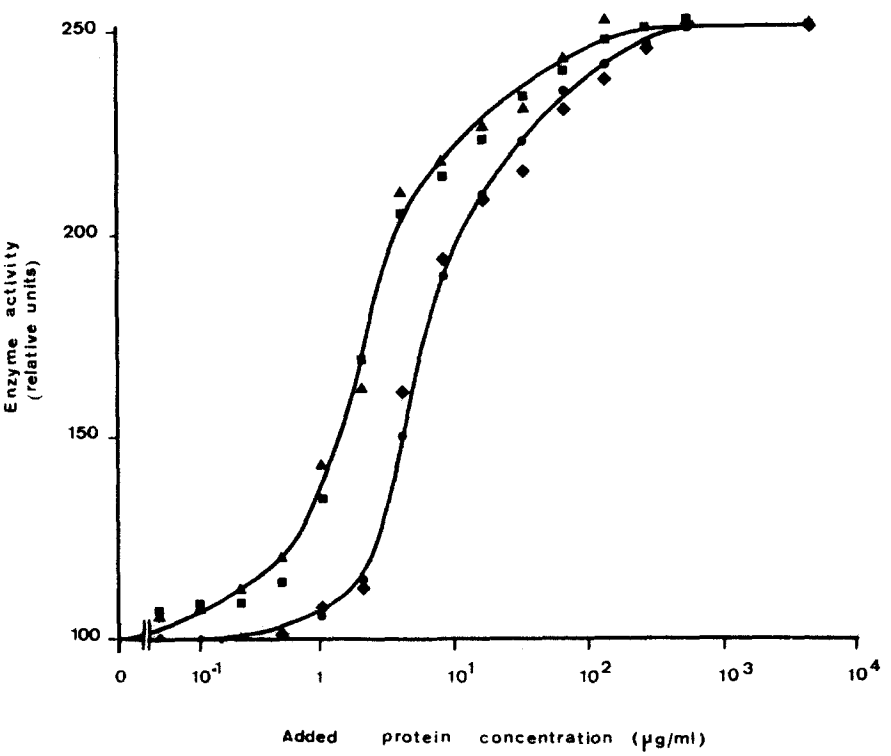

Fig. 1. Evolution of β-fructosidase activity when the enzyme assay is performed with the same standard amount of enzyme in presence of increasing concentrations of: albumin (▲——▲); ovalbumin (■——■); casein (●——●); rabbit immunoglobulins (◆——◆).

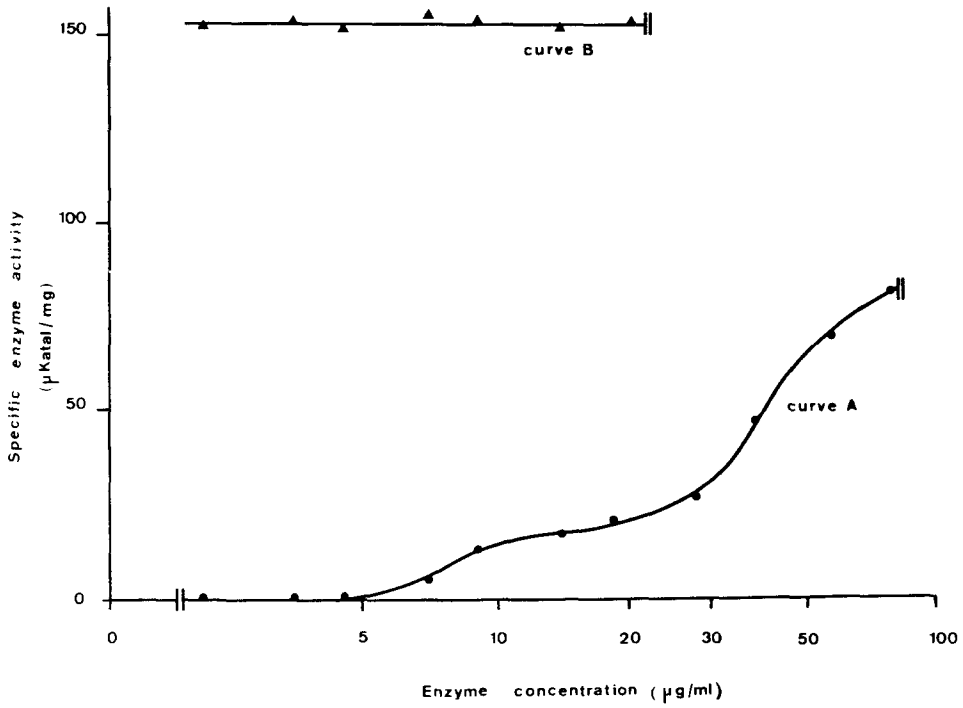

Fig. 2. Relation between specific β-fructosidase activity and enzyme concentration. The enzyme is diluted in 75 mM citrate phosphate buffer, pH 4.8, curve A (●———●); or in the same buffer containing 0.3 mg/ml albumin (final concentration), curve B (▲———▲). The limits indicated as ‖ on the curves correspond to the saturation of reducing sugars detection test [4].

containing albumin at the final concentration necessary to obtain the maximum increase in activity (curve B).

A specific activity of approx. 150 μkatal/mg was maintained when successive dilutions of enzyme were performed in buffer containing albumin. In the absence of albumin a decrease in specific activity upon dilution was observed and the curve A obtained had a sigmoidal shape.

Within the limits of our experiments (Fig. 2), the specific activity obtained in the presence of albumin is never reached without addition of this protein. Furthermore, no activity can be detected, when enzyme concentration is lower than 7 μg/ml, if β-fructosidase is assayed without albumin. The lower limit of detection is not reached when dilutions are performed in the presence of albumin. Higher limits of detection, corresponding to the saturation of reducing sugars detection test [4], are obtained with 18 μg/ml and 74 μg/ml of enzyme, respectively, with or without albumin.

Kinetic studies. The effects of pH and temperature on enzyme activity are quite similar with or without albumin, (data not shown) with identical optima.

K_m and V were determined for sucrose from Lineweaver-Burk plots; the values obtained for β-fructosidase diluted without albumin or in the presence of different amounts of this protein are represented in Fig. 3. Albumin did not affect V values appreciably, however the enzyme K_m decreases from 10 mM to

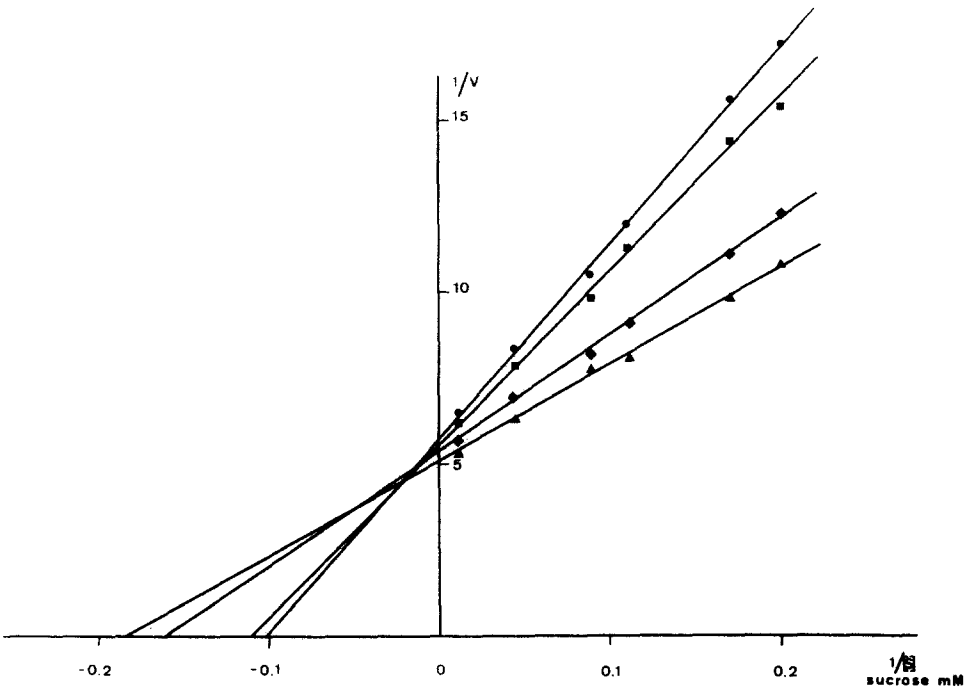

Fig. 3. Effect of albumin on the kinetic constants of β-fructosidase. The stock solution of enzyme is diluted and incubated with substrate at 37°C for 10 min, without albumin (●———●) or in presence of albumin at the final concentration of 0.003 mg/ml (■———■); 0.03 mg/ml (◆———◆) or 0.3 mg/ml (▲———▲).

5.4 mM, when the amounts of albumin added to the dilute enzyme increases from 0 to 0.3 mg/ml.

Adsorption of dilute β-fructosidase. All dilutes were carried out directly in test tubes from a standard stock solution of purified enzyme. Table I shows the β-fructosidase activity obtained from diluted solutions assayed in silicone-coated tubes or in buffer containing 0.5 M NaCl, or 0.5% Tween 20 or Tween 80. Under these various conditions, the activity of the dilute enzyme is approximately the same as that measured without any adsorption competitor. In each case presented, the enzyme activity is far from the values obtained when albumin is used, and furthermore activation by albumin is always observed (data not shown).

When diluted extracts were incubated without albumin, under conditions assumed to facilitate adsorption, we never observed any activity linked to the wall of the test tubes, even if measured in the presence of albumin. The dilute enzyme placed in a dialysis bag immersed in sucrose solution for enzyme assay could still be activated by albumin, but only when the protein was added to the bag itself (data not shown).

Protection of β-fructosidase from inactivation by albumin. When diluted samples are stored for 30 h at room temperature without albumin, the final β-fructosidase activity measured in the presence of albumin is the same as that of the samples maintained in presence of albumin during storage (data not shown). These results show that even diluted β-fructosidase has the same stabil-

TABLE I

Influence of some substances on the activity of higly diluted β-fructosidase. The enzyme diluted from a stock solution was incubated for enzyme assay in 75 mM citrate phosphate buffer/0.1 M sucrose, pH 4.8. The different substances tested were added to this reaction mixture at the concentrations indicated. The results presented here, were obtained with products that do not produce large interference with the detection of reducing sugars formed during the enzyme assay.

Added substances	Substances concentration	Relative activity
no addition		100
Bovine serum albumin	0.3 mg/ml	256
Ovalbumin	0.3 mg/ml	258
Rabbit immunoglobulins	0.6 mg/ml	253
Casein	0.6 mg/ml	254
EDTA	1 mM	130
Cystein	1 mM	118
Glycerol	1 mM	124
Ethylene glycol	1 mM	126
Tween 20	0.5%	103
Tween 80	0.5%	115
NaCl	0.5 M	108
Silicone	coating the test tubes	102

ity in the presence or absence of albumin.

The thermal or pH stability of β-fructosidase is unchanged by albumin: pH optima for preservation is 6.5 and the kinetics of thermal stability at 50°C are the same (data not shown).

Adsorption of β-fructosidase inactivators by albumin. Protectors of sulfhydryl groups or substances known to protect enzyme activity, such as glycerol, have a poor specific effect on diluted β-fructosidase activity (Table I). However, the slight activation observed with EDTA can be explained by the removal of a divalent ionic metal inhibitor. The adsorption by albumin of small molecules or ions acting as inactivators has been ruled out by placing enzyme and sucrose in a dialysis bag placed in a solution of albumin. In these defined conditions for enzyme assay, activation of β-fructosidase is not observed.

Albumin as a reagent to prevent dissociation of β-fructosidase. The effect of inactivation of some enzymes upon dilution has been explained by a dissociation into inactive products. Such an inactivating dissociation was shown to be reversed by either neutral proteins like albumin, (known to behave as polycations) or polycations themselves. From the data shown in Table II it is interesting to observe that the polycation protamine sulfate has the same activating effect as albumin on dilute solutions of enzyme. The highest activity is obtained with a concentration of 600 μg/ml protamine sulfate, as well as with 300 μg/ml albumin. The polyanion SDS has the same effect at a concentration of 300 μg/ml, while at 600 μg/ml it completely inactivates the enzyme. This effect with SDS is not observed with the other polyanions tested.

Inactive fragments of smaller molecular weight than the active enzyme are formed upon dilution, if dissociation is implicated in the inactivation of the enzyme. However, after extensive dialysis, highly diluted enzyme samples can still be activated by albumin and the activity obtained is the same as for an undialyzed solution (data not shown).

TABLE II

Influence of some polycations and polyanions on the activity of β-fructosidase highly diluted in buffer without albumin. Polyanions or polycations are added to substrate at the concentrations indicated. The β-fructosidase activity of the stock solution used here for enzyme assay is 221 in the presence of albumin when the activity of the same stock solution is taken as 100 in buffer alone.

Polyanion or polycation	Ion concentration μg/ml			
	75	150	300	600
Protamine sulfate	108	104	202	218
SDS	97	130	211	0
Dextran sulfate	107	102	108	108
Heparin	106	109	102	121

Two samples of diluted β-fructosidase were subjected to chromatography on ACA 54 Ultrogel. The first sample: A, was diluted in 85 mM citrate phosphate buffer/0.5 M NaCl, pH 6.5. The second sample: B, was diluted and chromatographed in the presence of albumin. Enzyme concentration, identical in both samples, was such that β-fructosidase activity in sample A, diluted and mea-

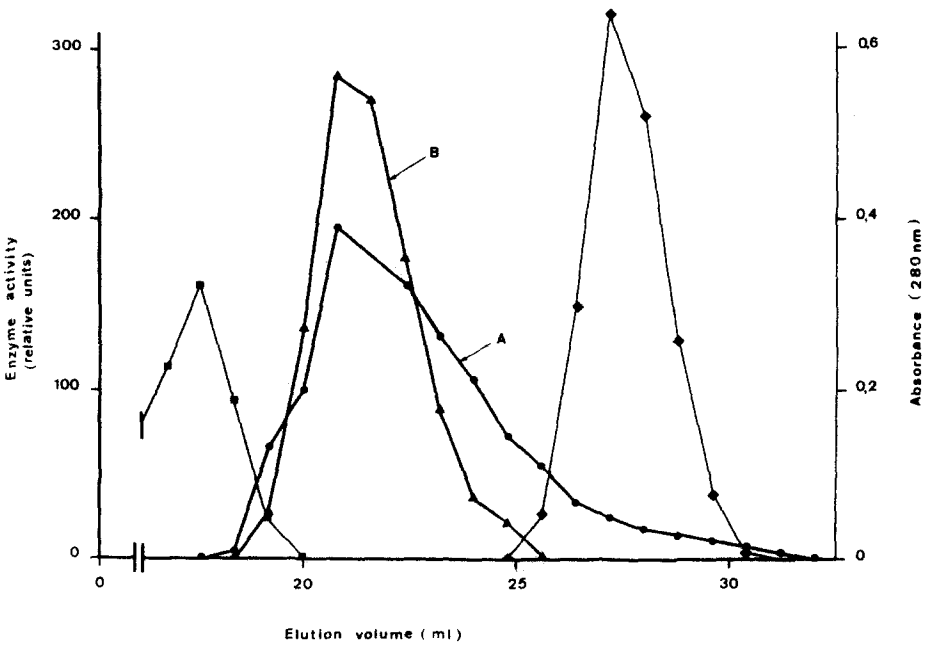

Fig. 4. Chromatographic analysis of β-fructosidase on a Ultrogel ACA 54 column 0, 90 cm × 50 cm. In the first case (curve A), the column was equilibrated and eluted with 85 mM citrate phosphate buffer/0.5 M NaCl, pH 6.5. A stock enzyme solution was diluted with the same buffer to 0.5 ml final sample volume. When β-fructosidase was chromatographed in presence of albumin (curve B) on the same column, dilution, equilibration and elution were performed in the same citrate buffer containing 0.3 mg/ml albumin. In both cases elution flow rate was 3.1 ml/h and 0.8-ml fractions were collected and finally assayed with sucrose for β-fructosidase activity, in the presence of albumin at a final concentration of 0.3 mg/ml. Elution profiles and volumes for protein standards, as indicated here for albumin (■———■) and cytochrome *c* (◆———◆), are the same in both conditions.

sured without albumin, was half the activity of B, diluted and measured with albumin. Standards of known molecular weight were subjected to chromatography under these conditions present the same profiles and elution volumes with and without albumin in the buffer.

The different fractions obtained from sample A (chromatographed without albumin), which could be activated in presence of albumin, are presented in Fig. 4, curve A. The total β-fructosidase activity of these fractions, measured independently or mixed together and then assayed, is identical to the activity recovered from sample B diluted and chromatographed in the presence of albumin. The elution volumes of β-fructosidase are 14.4 ml and 7.2 ml, respectively, with or without the presence of albumin in the dilution and chromatographic media. Both chromatography runs were performed in the presence of 0.5 M NaCl; this high ionic strength was used to avoid β-fructosidase adsorption onto the matrix (Faye, L. and Berjonneau, C., unpublished data).

Discussion

Many enzyme activities have been shown to be affected by protein concentration. Enzymes are sometimes inactivated when protein concentration is raised [6] but more generally the addition of proteins, particularly albumin, increases enzyme activity as demonstrated for α-amylase [7,8], trypsin [9] and, in our case, for β-fructosidase. Some workers have suggested that adsorption of dilute enzyme on glass or plastic surfaces can explain the inactivation, by loss of adsorbed molecules during transfer, or more specifically [10] by inactivation linked to contact with rough surface. In our study all dilutions and enzyme assays were carried out in the same test tubes and then an effect of albumin, as an agent preventing loss of enzymatic material during transfer, can be excluded. The presence of 0.5 M NaCl or 0.5% Tween 20 or Tween 80, or use of silicone-coated tubes, did not produce the same activation as albumin. Conversely, in conditions where adsorption could occur we did not detect β-fructosidase activity linked to the tubes when they were carefully washed. When all contact with glass was avoided during β-fructosidase assay, by maintaining the enzyme in a dialysis bag immersed in a sucrose solution, activation by albumin remained possible only if this protein was added within the dialysis bag itself: the effect of albumin cannot be explained by an inhibition of enzyme adsorption onto tube walls.

Inert exogenous proteins are often included in reaction mixture to protect enzymes from inactivation [11] or more precisely from endogenous inhibitors [12,13] which have sometimes been shown to be proteolytic enzymes [14]. Wallace [14] has shown that casein at 3% (w/v) was much more effective than a protease inhibitor such as phenylmethylsulfonyl fluoride to protect nitrate reductase isolated from maize roots. In the case of β-fructosidase, the increase of specific activity observed in the presence of albumin is neither due to stabilisation nor to protection from proteolysis.

Some sulfhydryl protector compounds and proteins [15], neutral detergents [16] or glycerol [17] have been used to protect β-amylase isolated from sweet potato. Addition of proteins [8] or poly(vinylpyrrolidone) [18] also increases urinary α-amylase activity. As with many glycosidases, β-fructosidase was

shown to be inactivated by several ions [19]; particularly metal ions, such as silver, partially [20] or completely [21,22] inactivate β-fructosidase from different species. The effects of EDTA (Table I) could lead to the hypothesis of the binding of inactivator ions by albumin. But when the enzyme assay was performed with a dialysis bag containing albumin immersed in a solution of β-fructosidase and sucrose, the enzyme activity measured was the same as that measured when the dialysis bag was free from albumin: β-fructosidase activation is not linked to the adsorption of diffusible inactivators. Furthermore, with the purified preparation of enzyme used for this study the probability of contamination by endogenous inhibitors, as described for coumarate coenzyme A ligase of sween potato roots [12], is considerably reduced.

Reversible loss of specific enzyme activity upon dilution has been explained by dissociation into inactive products for several enzymes such as hyaluronidase [23], β-glucuronidase [24], aldolase, lactic dehydrogenase, α- and β-amylases [10]. These dissociations linked to a loss of specific activity are prevented by albumin, ovalbumin or other substances, the common property of these compounds being their polycationic nature. Polyanions were shown to act reversibly as inhibitor. The reactivation of dilute β-fructosidase can also be obtained by protamine sulfate with the same final value as for albumin. SDS also increased β-fructosidase activity while polyanions are known to counteract the biological effects of polycations or basic proteins. Consequently, whether the inhibition of β-fructosidase upon dilution is due to dissociation of the enzyme or to other mechanisms can be elucidated by molecular weight criteria: dilute solutions of β-fructosidase extensively dialysed are still capable of being activated by albumin; then, if dissociation occurs upon dilution, the size of subunits formed is higher than the dialysis limits.

The results obtained from chromatographic analysis of β-fructosidase, performed with or without albumin, are not compatible with formation of small inactive fragments from the dilute enzyme, which should have been separated on a basis of molecular weight, and then inactivation of β-fructosidase upon dilution cannot be explained by dissociation of the enzyme. So, the decrease of β-fructosidase specific activity upon dilution is not linked to a dissociation of the enzyme, but rather to an apparent molecular weight heterogeneity of β-fructosidase. Such a decrease of apparent molecular weight upon dilution has already been described for bovine hepatic glutamic acid dehydrogenase but without loss of enzyme activity [25]. As for potato β-amylase [16], changes in molecular conformation may explain the loss of β-fructosidase activity on dilution. Then, it should be assumed that certain linkages or interactions of the enzyme molecules which are essential for activity can be released upon dilution. Whatever may be the nature of these linkages, they are shown here to be involved in active structural conformation of β-fructosidase and are maintained in the presence of either proteins or some polyanions and polycations, the action of which decreases during dilution.

Acknowledgements

This work was supported by the Centre National de la Recherche Scientifique (L.A. 203). I wish to thank Dr. J.P. Salier for helpful discussions.

References

1 Faye, L. and Berjonneau, C. (1979) Biochimie 61, 51—59
2 Aspberg, K. and Porath, J. (1970) J. Acta Chem. Scand. 24, 1839—1841
3 Harboe, N. and Ingild, A. (1973) Scand. J. Immunol. 2 suppl. 3, 161—169
4 Nelson, N. (1944) J. Biol. Chem. 153, 375—380
5 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
6 Faye, L. (1977) Biochimie 59, 345—350
7 Irie, A., Hunaki, M., Bando, K. and Kawai, K. (1972) Clin. Chim. Acta 42, 63—66
8 Hao, K., Takeuchi, T., Sato, S. and Sugimura, T. (1977) Clin. Chim. Acta 79, 75—80
9 Shora, W., Fortner, G.G. and Forstner, J.F. (1975) Gastroenterology 68, 470—479
10 Bernfeld, P., Berkeley, B.J. and Bieber, R.E. (1965) Arch. Biochem. Biophys. 111, 31—38
11 Dalgardo, L. and Birt, L.M. (1962) Biochem. J. 83, 195—201
12 Rhodes, M.J.C. and Wooltortorn, L.S.C. (1973) Phytochem. 12, 2381—2386
13 Schrader, L.E., Cataldo, D.A. and Peterson, D.M. (1974) Plant Physiol. 53, 688—690
14 Wallace, W. (1975) Plant Physiol. 55, 774—777
15 Walker, G.J. and Whelan, W.J. (1960) Biochem. J. 265—270
16 Takeda, Y. and Hizukuri, S. (1972) Biochim. Biophys. Acta 268, 175—183
17 Banks, W. and Greenwood, C.T. (1969) Stärke 21, 177—180
18 Irie, A., Hunaki, M., Bando, B. and Kawai, K. (1974) Clin. Chim. Acta 51, 241—245
19 Matsushita, K. and Uritani, I. (1974) Plant Physiol. 54, 60—66
20 Arnold, W.N. (1965) Biochim. Biophys. Acta 110, 134—147
21 Cooper, R.A. and Greenshields, R.N. (1964) Biochem. J. 92, 357—364
22 Nakagawa, H., Kawasaki, Y., Ogura, N. and Takehana, H. (1971) Agr. Biol. Chem. 36, 18—26
23 Bernfeld, P., Tuttle, L.P. and Hubbard, R.W. (1961) Arch. Biochem. Biophys. 92, 232—240
24 Reithel, F.J. (1963) Adv. Protein Chem. 18, 123—129
25 Fisher, H.F., Cross, D.G. and McGregor, L.L. (1962) Nature 196, 895—900