

Immobilization of Potato Acid Phosphatase on Succinamidopropyl Glass Beads for the Dephosphorylation of Bovine Whole Casein

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ABSTRACT

Potato acid phosphatase was immobilized on succinamidopropyl-derivatized glass beads for use in removing phosphate groups from bovine whole casein. Controlled pore glass beads (with and without glyceryl coating) were silanized and succinylated. The enzyme was covalently bound to derivatized glass beads through carbodiimide coupling. The pH optima for the immobilized enzyme remained at pH 5.5 for *p*-nitrophenyl phosphate and pH 6.0 to 6.5 for casein substrates. Activities of acid phosphatase (pH 5.5, *p*-nitrophenyl phosphate substrate) immobilized on the plain and glyceryl-coated controlled pore glass beads were 14 and 4%, respectively, of the free enzyme. When whole casein was used as substrate, the 15 to 20 min enzyme activity of free enzyme was 16% of its activity on *p*-nitrophenyl phosphate and after 4 h, 14% of its original rate on casein. After 18 h of incubation, the activity of the immobilized enzyme was 36 to 40% of its rate at 4 h. A 70% dephosphorylation of whole casein was attained using a concentration of 2.6 mg enzyme controlled pore glass beads complex per milligram of whole casein at pH 6.5, 37°C, for 18 h.

(Key words: acid phosphatase, casein, dephosphorylation)

INTRODUCTION

Removal of phosphate groups from casein significantly alters the properties of these milk

proteins in ways that may be of interest to the food industry. As examples, dephosphorylated (DP) casein has altered physical properties including 1) reduction of calcium binding by DP α_s -caseins and β -casein (2, 28, 29); 2) increase in calcium sensitivity by DP α_s -caseins (2, 4); 3) loss in the ability of DP β -casein to precipitate in the presence of calcium (28); 4) disruption in the interactions between casein components (4, 16, 25) and their stability (16, 18, 25, 29); 5) increase in abnormal micelles (3, 4, 18, 25); 6) extended rennet clotting time (11, 15, 21, 26); and 7) reduced curd tension (14, 21, 26, 29). Acid dispersions of DP bovine casein have a very fine microstructure and greater pepsin hydrolysis rates than native casein (13). These dephosphorylation characteristics may be advantageous in the use of bovine whole casein in infant formulas. Dephosphorylation of casein is catalyzed by phosphoprotein phosphatases (EC 3.1.3.16) (2, 3, 4, 16, 23, 26, 27, 28, 29) and acid phosphatases (APase) (EC 3.1.3.2) (5, 11, 13, 14, 18, 21, 25). Although spleen phosphoprotein phosphatases required activating agents (ascorbic acid or 2-mercaptoethanol) for maximum activity, potato acid phosphatase (pAPase) is more desirable because it requires no cofactors, is active at pH 7 (where native and DP caseins are readily soluble), and is not proteolytic toward casein (5, 11). Although methods are available to dephosphorylate casein in small quantities (5), the use of soluble enzymes is not feasible when larger quantities are required. Recovery and reuse of expensive enzymes may make it economically feasible for DP caseins to be used in the food industry.

Immobilization of APase with glutaraldehyde has been published in two studies (14, 24), which reported 19 to 44% and 60% reduction in activity with *p*-nitrophenyl phosphate (pNPP) substrate when APase was immobilized on acrylamide beads (24) and porous glass beads (14), respectively. The APase bound to glass beads had negligible activity toward skim

milk (14). Immobilization of enzymes with carbodiimide has been suggested for those that are optimally active at acid pH (22). Immobilization has been used successfully to bind up to 20 mg of protein/g of glass beads for a variety of enzymes (6, 7, 12).

In this study, we report the covalent attachment of pAPase to succinamidopropyl glass beads, the determination of activity rates with pNPP substrate, and the comparison of the ability of the free and immobilized enzymes to dephosphorylate whole casein.

MATERIALS AND METHODS

Material

The following chemicals were obtained from Sigma Chemical Co.¹ (St. Louis, MO): 3-aminopropyltriethoxysilane; controlled pore glass beads (pore diameter 2000Å, mesh 120 to 200); 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; glyceryl-coated controlled pore glass (pore diameter 2000Å, mesh 120 to 200); pNPP (Sigma 104 phosphatase substrate); and 2, 4, 6-trinitrobenzenesulfonic acid. Accugel-40 was obtained from National Diagnostics (Manville, NJ), Spectra/Por[®] 1 from Spectrum Medical Industries, Inc. (Los Angeles, CA), pAPase from Calbiochem (La Jolla, CA), and succinic anhydride from Aldrich Chemical Co., Inc. (Milwaukee, WI). Other chemicals used were of analytical reagent grade.

Casein Preparation

Fresh milk, obtained from a commercial dairy herd, was warmed to 40°C for cream removal (De Laval Separator Co., Poughkeepsie, NY). The skim milk was diluted 1:1 with distilled water, adjusted to pH 4.6 with 4 N HCl with stirring, and stored overnight at 4°C to precipitate the casein. Acid casein was washed several times with distilled water at pH 4.6 to remove residual whey, filtered in felt bags, and stored wet at -20°C. Sodium caseinate was prepared by suspending acid casein in water titrated to pH 7 to 7.5 with 4 N NaOH.

¹Reference to brand or firm name does not constitute endorsement by the United States Department of Agriculture over others of a similar nature not mentioned.

The protein was lyophilized and stored at -20°C.

Preparation of Supports

Succinamidopropyl-derivatized supports were prepared (12, 22) from controlled pore glass beads (CP) and glyceryl-coated controlled pore glass beads (GCP). Beads were washed in 5% nitric acid for 60 min at 80°C and rinsed extensively with distilled water between each of the following steps. 1) Clean beads were treated with 10% aqueous 3-aminopropyltriethoxysilane (20 ml/g support) pH 3 to 4 at 75°C for 2 h. This treatment placed a monolayer of silane on the beads' surface. 2) Beads were filtered, dried overnight in a 115°C oven, and 3) mixed overnight at approximately 20 to 23°C with 1 g aqueous succinic anhydride/g support, pH 6.0 (8, 22). Step 3 was repeated until the air-dried beads gave a negative trinitrobenzenesulfonic acid test (8), which indicated that all of the alkylamine sites had reacted with carboxyl groups. 4) The derivatized beads were activated by treatment with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (200 mg carbodiimide/g support), pH 4 for 2 h at approximately 20 to 23°C (12), prior to 5) exposure to a dilute enzyme solution, (4 mg/ml, total 32 mg protein/g support), pH 4.5 for 2 d at 4°C. The immobilized enzyme-support complexes were rinsed and stored in water at 4°C. The immobilized enzyme-support complex are identified in the text according to their glass supports; therefore, CP and GCP refer to enzyme bound to plain controlled pore glass beads and glyceryl-coated controlled pore glass beads, respectively.

Bound Enzyme

The amino acid profile and the amount of enzyme bound to the supports were determined by amino acid analysis (9) and compared to the free enzyme. Proteins on the supports were digested from the beads and hydrolyzed with 4.7 N HCl at 115°C for 24 h under nitrogen evacuation. The hydrolyzed amino acids were dissolved in .2 N sodium citrate buffer and quantitated with a Beckman 119 CL amino acid analyzer (Beckman Instruments, Palo Alto, CA). The amino acid profile of the protein hydrolyzed from the beads was expressed as molar ratios based on arginine = 1.0 and was

compared with the profile of the free enzyme similarly hydrolyzed.

Enzyme Activity

Enzyme activity of free and immobilized pAPase were determined with both pNPP and bovine whole casein as substrates (5) and expressed in units defined as nanomoles of product (*p*-nitrophenol or phosphate) released per minute.

p-Nitrophenyl Phosphate Substrate. The reaction mixture contained pNPP (5 μ mol/ml) in .1 M acetate buffer at pH 5.0, 5.5, 6.0, 6.5, or 7.0. Free (2 μ g) or immobilized (10 to 15 mg CP or GCP) enzyme was added to 5 ml of pNPP-acetate buffer and incubated at 37°C in a shaker water bath (125 rpm) for up to 120 min. Filtered aliquots (.5 ml) were removed at 15-min intervals. The reaction was quenched with 1.5 ml .25 M NaOH and absorbency read at 410 nm. The specific enzyme activity was calculated based on the extinction coefficient of 1 nmol *p*-nitrophenol/ml = .0162 and expressed in units per microgram of free or bound enzyme. At the end of the incubation, the glass beads were recovered by filtration, extensively washed in water, and lyophilized to obtain the weight of CP and GCP.

Casein Substrates. The reaction mixture contained casein (3 mg/ml) in .1 M acetate buffer at pH 5.5, 6.0, 6.5, and 7.0. Two micrograms of free enzyme were added to 1 ml of casein-acetate buffer and incubated at 37°C for 15 min for pH 5.5 and 6.0 and 20 min for pH 6.5 and 7.0. The reaction was stopped by immersion in a boiling water bath for 10 min. Equal volumes of sample and 20% trichloroacetic acid (TCA) were mixed and centrifuged for 10 min at 8800 \times g (Eppendorf, Brinkmann Instruments, Inc., Westbury, NY). Aliquots of the supernatant were then taken for phosphate assay (below). The specific activity (15- to 20-min rates) for free enzyme was calculated in units per microgram of protein.

Fifteen milliliters of casein reaction mixture (45 mg) in .1 M acetate buffer were adjusted to pH 5.5, 6.0, 6.5, and 7.0. Enzyme (50 mg CP or GCP or 15 μ g free enzyme) was added to 15 ml of buffered casein and incubated at 37°C in a shaker water bath (150 rpm) for 4 h. Glass beads were removed by filtration, and the reaction mixture was heated in boiling water to

inactivate any enzyme present. The solution was dialyzed 2 d against water at pH 7.5 at 4°C to remove free phosphate and the retentate lyophilized. Organic phosphate per milligram of casein was determined (below) to calculate the amount of phosphate removed during incubation. Specific enzyme activities were expressed in units per microgram of free or bound protein. The recovered glass beads were rinsed extensively with distilled water and lyophilized to obtain the weight of CP and GCP.

Phosphate content of the supernatants and caseins was determined using a modification of Sumner's (19) procedure. Lyophilized casein samples (10 mg) were digested in 7.5 N sulfuric acid. Hydrogen peroxide (30%) was added two to three drops at a time until the solutions cleared upon heating. Aliquots of digested casein (diluted to 10 ml) or TCA-precipitated supernatants were treated with 2.5% ammonium molybdate, 7.5 N sulfuric acid, and 10% ferrous sulfate, color developed for 30 min, and the absorbency read at 650 nm.

Extended Casein Incubation

Aliquots of wet CP (1.2 g) and GCP (.58 g) were transferred to dialysis membranes containing 300 and 100 ml, respectively, of a pH 6.5 acetate-buffered casein solution (3 mg/ml). Dialysis membranes were submerged in 18 L of deionized water at pH 6.5 and shaken (150 rpm) at 37°C. After 24 h, the retentate was filtered to remove glass beads and lyophilized. Organic phosphate per milligram of casein was determined as described earlier. Recovered glass beads were rinsed extensively with distilled water and lyophilized to obtain the weight of CP and GCP.

Aliquots of wet CP and GCP (estimated at .78 g each) were packed into separate 1 \times 30-mm jacketed glass columns. Casein (300 mg) was dissolved in 100 ml .1 M acetate buffer at pH 6.5 and circulated (4.5 ml/min) (Peristaltic Pump P-1, Pharmacia, Sweden) continuously in a reverse flow pattern through the column (approximately 30 ml capacity) to an attached dialysis membrane (approximately 70 ml capacity), which was submerged in 3 L of deionized, distilled water at pH 6.5 (replaced at 12 h). An agitating water bath at 37°C was used 1) to maintain temperature of water

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pumped through the jacketed column and 2) to hold the beaker in which the dialysis membrane was placed. After 18 h, the incubation was terminated because of packing of glass supports and precipitating casein, which clogged pump lines and column filters. Precipitated casein and solution that had collected in the dialysis membrane and solution drained from the column were combined, heated in a boiling water bath for 10 min, and dialyzed overnight in water at pH 7.0, 4°C. The retentate was lyophilized and organic phosphate per milligram of casein was determined. Due to extensive precipitation of casein on the glass beads, the weight of CP and GCP used for this trial was estimated from earlier weights obtained for wet volumes of beads.

Urea-Polyacrylamide Gel Electrophoresis

Electrophoresis of casein samples was performed using an EC Apparatus Cell and 7% urea-polyacrylamide gel (10) without the stacking gel. A solution of 50 μ l .025 M Tris and .2 M glycine buffer, 30 μ l glycerol, 10 μ l .25%

bromophenol blue, and 10 μ l 2-mercaptoethanol was used to dissolve 1 mg casein. A 1:10 dilution of Tris-glycine solution was used as the running buffer. Current was applied at 100 V and increased 50 V every 10 min to 300 V. The gel was maintained at 14°C for the duration of the run. Protein bands were stained overnight in .25% Coomassie brilliant blue R in 4.5% methanol-8% acetic acid and destained in 10% methanol-7% acetic acid in water.

Statistics

The SAS Software System (17) was used to evaluate enzyme kinetics using a general linear model and to compare means using Bonferroni *t* test ($\alpha = .05$).

RESULTS

Enzyme Bound to Support

The amino acid profiles of the proteins removed from CP and GCP were compared with the free pAPase profile (Table 1); molar ratios in 13 of the 17 amino acids quantitated were

TABLE 1. Amino acid composition of proteins hydrolyzed from free and immobilized potato acid phosphatase (pAPase) preparations.

	Free pAPase	pAPase-Controlled pore glass beads	pAPase-Glyceryl-coated controlled pore glass beads
Protein level	.890 mg/ml	.553 \pm .103 mg/g CP	.641 \pm .159 mg/g GCP
Amino acid ¹			
Asp	2.65	3.03	2.96
Thr	1.65	1.58	1.72
Ser	1.22	2.18	2.81
Glu	3.66	3.75	3.49
Pro	1.31	1.24	1.26
Gly	.88	2.59	3.19
Ala	2.01	1.91	1.95
1/2 Cys	1.14	0	0
Val	1.39	1.42	1.35
Met	.17	.08	.13
Ile	.69	.93	.98
Leu	2.73	2.44	2.37
Tyr	.87	.74	.81
Phe	1.17	1.08	1.05
Arg	1.00	1.00	1.00
Lys	2.54	1.77	1.69
His	.69	.79	1.03

¹Amino acids are expressed as molar ratios based on Arg = 1.00. Asp = Aspartic acid, Thr = threonine, Ser = serine, Glu = glutamic acid, Pro = proline, Gly = glycine, Ala = alanine, 1/2 Cys = half-cystine, Val = valine, Met = methionine, Ile = isoleucine, Leu = leucine, Tyr = tyrosine, Phe = phenylalanine, Arg = arginine, Lys = lysine, His = histidine.

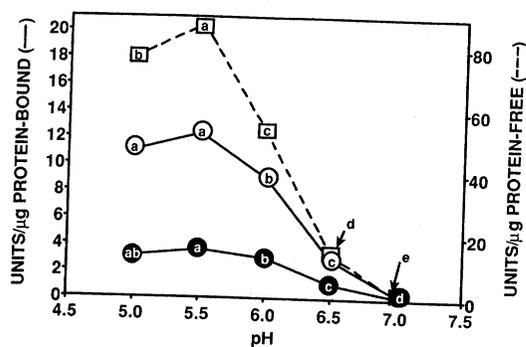


Figure 1. Specific activity at different pH of free and immobilized potato acid phosphatase (pAPase) with *p*-nitrophenyl phosphate as the substrate and incubated at 37°C. Symbols represent: free enzyme (□), pAPase-controlled pore glass beads (CP) (○), and pAPase-glycero-coated controlled pore glass beads (GCP) (●). Enzyme activity is expressed in units (1 nmol of *p*-nitrophenol released/min)/μg protein, free or bound. Activity means at different pH within each support are compared using Bonferroni *t* test. Means with the same letter are not significantly ($P < .05$) different.

very similar. Because of the limited quantity of immobilized enzyme available for digestion and analysis, the ratios of common contaminants, serine and glycine, were higher for the immobilized enzyme than for pure enzyme preparations; however, the analyses were based only on triplicate 24-h hydrolysates. Isoelectric focusing (not shown) of the commercial enzyme located the majority of protein and acid phosphatase activity at pI to 5.2 to 5.3. A minor band with some activity was located at pI of 7.3. The focusing results showed that the commercial enzyme preparation was of high purity and the similar amino acid profiles indicated that the protein bound to the glass beads was pAPase. The immobilization procedure bound the same amount of enzyme to the glass beads regardless of the glycerol coating.

Enzyme Activity, *p*-Nitrophenyl Phosphate Substrate

Immobilization of pAPase on succinamidopropyl glass resulted in 14 and 4% of the activity of the free enzyme with *p*NPP at pH 5.5 for CP and GCP, respectively. Although porous supports bound the same amount of

enzyme, the activity for CP was 3 to 3.5 times higher than GCP (Figure 1).

The pH optimum of 5.5 for *p*NPP substrate did not change when the enzyme was immobilized (Figure 1). The activity for CP was highest at pH 5.0 to 5.5 but significantly ($P < .05$) decreased at pH ≥ 6.0 . The GCP was less affected by pH changes with slight changes in activity between pH 5.0 to 6.0, but activity was lower at pH 6.5 and 7.0. When the pH was increased from 5.5 to 6.5, free enzyme activity was reduced 84% and immobilized enzyme activity on porous beads decreased 70 to 77% (Figure 1). The enzyme activity for free pAPase decreased 84% when the substrate was changed from *p*NPP at pH 5.5 to whole casein at pH 6.5 (rate calculated after 15 to 20 min) (Table 2).

Enzyme Activity, Casein Substrate

Free Enzyme. Specific activities (casein substrate) were calculated for the 15- to 20-min and 4-h rates for the free enzyme (Table 2) and were based on $7.17 \pm .11$ μg phosphate/mg bovine whole casein. The maximum free enzyme activity occurred at pH 6.0 and decreased 23% at pH 6.5. Although the pH optimum for free enzyme was 6.0, pH 6.5 was selected for casein studies, as the DP caseins were more soluble at the higher pH, which was still within the immobilized enzyme's optimal activity range. The activity of the free enzyme

TABLE 2. Specific activities (units/μg enzyme) for free potato acid phosphatase with *p*-nitrophenyl phosphate (*p*NPP) and whole casein as substrate.

pH	Specific activity ¹			%DP ² after 4 h
	<i>p</i> NPP	Casein		
		15 to 20 min	4 h	
5.0	76.9 ^b			
5.5	87.0 ^a	16.6 ^b	1.83 ^c	64.3
6.0	52.7 ^c	18.5 ^a	2.24 ^a	78.8
6.5	13.8 ^d	14.3 ^c	1.97 ^b	69.3
7.0	.71 ^e	8.2 ^d	1.15 ^d	40.6

^{a,b,c,d,e}Activity means at different pH within each support are compared using Bonferroni *t* test. Means with the same letter are not significantly ($P < .05$) different.

¹One unit of activity is defined as 1 nmol product (*p*-nitrophenol or phosphate) released/min.

²Dephosphorylated.

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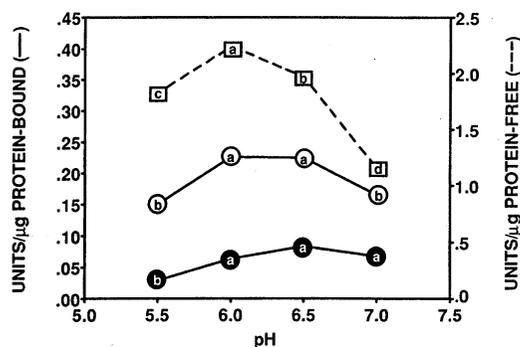


Figure 2. Specific activity at different pH of free and immobilized potato acid phosphatase (pAPase) with whole casein as the substrate after 4 h of incubation at 37°C. Symbols represent: free enzyme (□), pAPase-controlled pore glass beads (CP) (○) and pAPase-glyceryl-coated controlled pore glass beads (GCP) (●). Enzyme activity is expressed in units (1 nmol phosphate released/min)/μg protein, free or bound. Activity means at different pH within each support are compared using Bonferroni *t* test. Means with the same letter are not significantly ($P < .05$) different.

decreased 7-fold after 4 h of incubation with casein substrate.

Immobilized Enzyme. Immobilization broadened the pH optimum for the enzyme with whole casein as substrate. Controlled pore glass beads had its highest activity at pH 6.0 to 6.5 with a 27% decrease in activity between pH 6.5 to 7.0 (Figure 2). Glyceryl-coated controlled pore glass beads had its highest activity at pH 6.0 to 7.0.

Immobilization of pAPase reduced specific activity further. After 4 h of batch incubation, the free enzyme had an activity of 1.97 units/μg protein (Table 2), and GCP and CP had activities of .084 and .225 units/μg protein, respectively (Table 3). The activity decreased 60 to 64% until stabilizing at 18 h. The activities for 18 and 24 h were very similar for GCP and CP.

Dephosphorylation of Whole Casein

Urea-Polyacrylamide Gel Electrophoresis. The incubation of immobilized pAPase in a casein solution for 4 h resulted in a maximum dephosphorylation of 15.2% for CP at pH 6.0 and 4.6% for GCP at pH 6.5. Urea-PAGE patterns (Figure 3) for incubated casein at various pH showed that control casein (lanes 1 and

6, no enzyme during incubation) had major zones for α_{s1} - and β -casein fractions, a minor doublet for κ -casein and α_{s2} -casein, and a minor zone for β -casein fragments. Incubation with CP resulted in zones of DP caseins with slower mobilities than the native caseins (lanes 2 to 5). There was a maximum of three to four zones for DP α_{s1} -casein and three zones for DP β -casein. The heaviest zones for DP caseins were visible at pH 6.0 (lane 3), followed by pH 6.5 (lane 4). Faint DP zones were noted at pH 5.5 and 7.0 (lanes 2 and 5, respectively). When casein was incubated with GCP (not shown), a very faint zone for DP β -CN appeared, but no DP α_{s1} -casein zones were observed.

The urea-PAGE profile (Figure 4) for long-term incubated casein (before incubation, lane 1; after incubation, lanes 2 and 3) demonstrated the disappearance of the native casein fractions and the appearance of the slower migrating DP protein zones. After 24 h of incubation with CP (lane 2), casein had very little of the native α_{s1} and β -casein zones remaining, and the intensity of the DP zones increased as the percentage dephosphorylation increased. With the 24-h GCP incubation (lane 3), a urea-PAGE profile with a strong remaining native α_{s1} -casein zone but a weaker β -casein, and a concomitant accumulation of DP caseins, is apparent. Incubation of casein with either CP or GCP resulted in maximally DP β -casein, which migrated in the region of κ -casein, making identification of the latter difficult.

Ratio of Enzyme-Support Complex to Substrate. An increase in weight ratio of CP and GCP to substrate, an increase in incubation times, and the inclusion of dialysis during the incubation resulted in higher percentages of DP casein (Table 3). The 4-h batch incubation used a ratio between 1 to 1.2 mg of support/mg casein (except CP at pH 7.0, which was .75 mg support/mg casein) and resulted in 13 and 4% DP casein for CP and GCP, respectively. The 24-h batch incubation ratios were increased 30% (CP) and 79% (GCP) and resulted in 2.7- and 5.6-fold increase in DP casein, CP and GCP respectively. During the 18-h column incubation, the ratio was increased to 2.6 mg CP or GCP/mg casein, which resulted in a 100% (CP) and a 32% (GCP) increase in DP casein over the 24-h preparation. Although the casein solutions were contaminated during the 18-h incubation study, enzymatic dephosphorylation

still occurred on intact or proteolyzed casein fragments (PAGE not shown).

Despite different incubation methods for the 18- and 24-h studies, the specific activities rates for type of support were similar (Table 3). Using the activity rates from the 24-h batch incubation and the proportional increase in the support complex:substrate ratio, calculated DP casein yields for column incubation were close to actual yields. This suggested that the ratio of CP:substrate or GCP:substrate influenced percentage DP casein more than the method of incubation.

DISCUSSION

Enzyme Bound to Support

Immobilization of pAPase through carbodiimide coupling to succinamidopropyl glass beads resulted in low quantities of enzyme being bound, .5 mg protein/g glass beads. Swaisgood's group (6, 12), however, has reported considerably higher binding of the enzymes, sulfhydryl oxidase (2 to 10 mg/g), and microbial pronase (20 mg/g) to porous glass beads.

TABLE 3. Specific activity (units/ μ g bound enzyme), percentage of dephosphorylated (DP) whole casein, and support:substrate ratios for 4, 18, and 24 h of incubation at 37°C, pH 6.5 with immobilized potato acid phosphatase (pAPase)

	pAPase-Glycerol coated controlled pore glass beads	pAPase-Controlled pore glass beads
4-h Batch		
Specific activity	.084	.225
Support:substrate	1.08	1.02
% DP	4.3	12.8
24-h Batch		
Specific activity	.034	.081
Support:substrate	1.93	1.33
% DP	24.3	35.0
18-h Column		
Specific activity	.040	.103
Support:substrate	2.60	2.60
% DP	32.1	70.0

Enzyme Activity

pH Optima. The optimum activity of pAPase is at pH 5.0 to 6.0 with pNPP as the substrate (1, 5). When α_{s1} -casein is used as substrate, the optimum pH was 5.8 to 7.0 with less than 10% variation (5). In this study, the pH optima for free and immobilized pAPase were very similar. Optimal activities with pNPP as the substrate were at pH 5.0 to 5.5 and optimal activities with whole casein as substrate were at pH 6.0 to 6.5. Carbodiimide coupling of pAPase did not result in shift of the pH optima for the substrate, pNPP, from pH 5.5 to 4.5, as was observed when glutaraldehyde was used to immobilize wheat germ and sweet potato APase on porous glass beads (14).

Glutaraldehyde has often been the preferred immobilization technique for enzymes operating at basic pH optima. The pH optima shift for enzymes attached by glutaraldehyde may have been due to the aldehyde linkage between the glass derivative and the epsilon amino groups of lysine on the enzyme. Another suggested reason for the pH shift had been the polycationic nature of glass beads that attracted OH⁻ and formed a microenvironment around the enzyme (14). The glutaraldehyde linkage was undesirable because it shifted the APase pH optimum further to the acid side. This pH range was unsuitable for working with DP casein, which was insoluble below pH 6.0. However, carbodiimide coupling of the enzyme, through the carboxyls of the glass derivative and the free amino groups of the enzyme, did not result in a pH shift of the pAPase, as reported in this study, thereby providing an immobilized enzyme that was suitable for the DP of whole casein. This observation is consistent with reports of immobilization of other acid-active enzymes (22) coupled by the same procedure (7, 20).

p-Nitrophenyl Phosphate Substrate. Immobilization of enzymes usually results in decreased activity for the enzyme. In this study, immobilized pAPase had 14 and 4% the activity of the free enzyme with pNPP substrate at pH 5.5 (CP and GCP, respectively). The activity of wheat germ APase bound to acrylamide beads with glutaraldehyde was 56 to 81% of the activity of the free enzyme using pNPP substrate (24). Acid phosphatase bound to the Dowex resins had no activity after immobilization, although the same amount of protein

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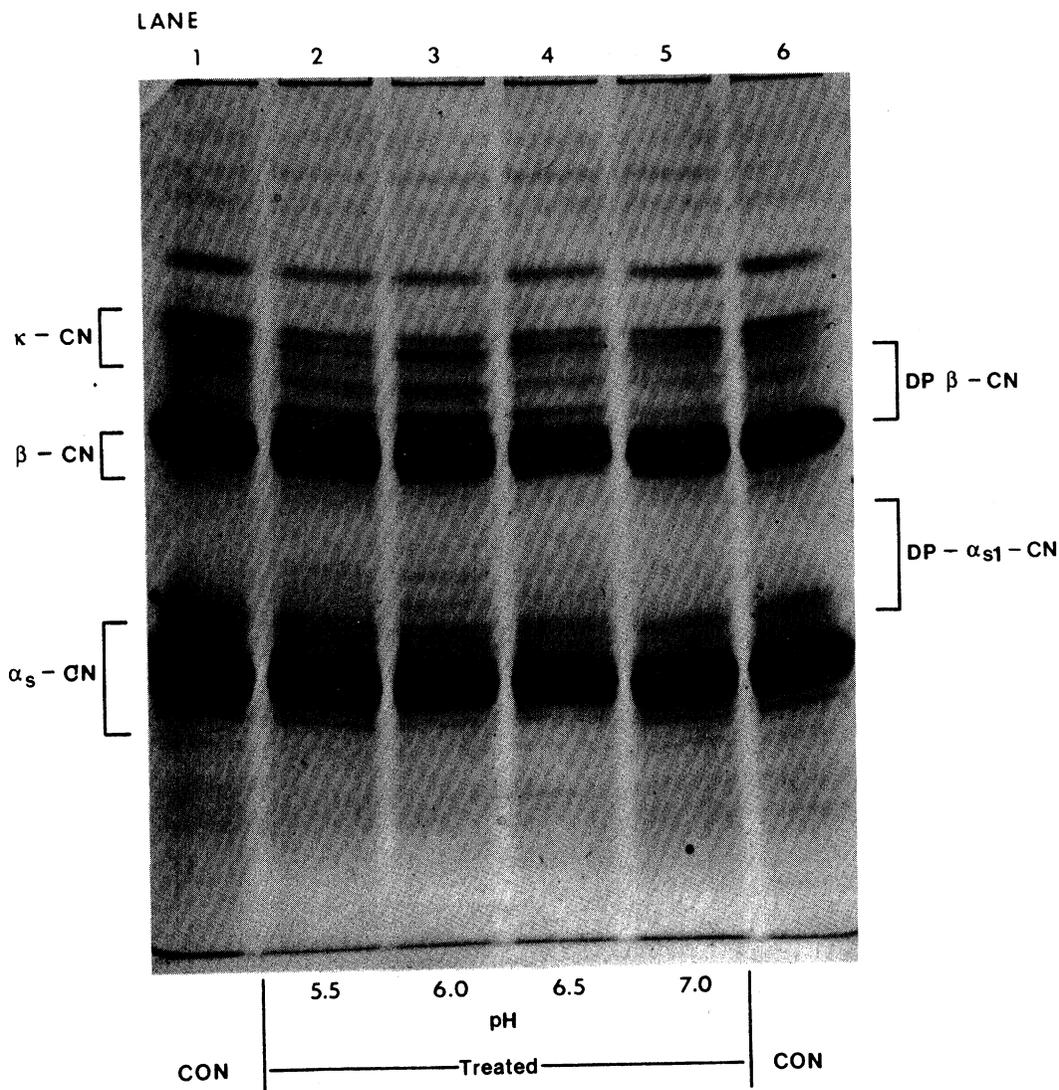


Figure 3. Urea-PAGE (7% acrylamide gel) profile of partial dephosphorylated (DP) bovine whole casein (CN) after incubated 4 h at 37°C at different pH with potato acid phosphatase immobilized on controlled pore glass beads. Samples (300 μ g/slot) are lanes 1 plus 6) casein, no enzyme during incubation (CON), and lanes 2 to 5) casein incubated with immobilized enzyme in buffer at 2) pH 5.5, 3) pH 6.0, 4) pH 6.5, and 5) pH 7.0.

bound to the glass beads had activities 38 and 44% of the free enzyme when *p*NPP was the substrate (wheat germ and sweet potato, respectively) (14).

Casein Substrates. Changing from *p*NPP to casein substrates resulted in decreased pAPase activity similar to that reported in another study (5). Our free pAPase activity decreased 6-fold

or 84%. Bingham (5) reported a 7- to 8-fold decrease in maximum velocity (V_{max}) when changing substrate from *p*NPP to α_{s1} -casein, though the Michaelis-Menten constants were similar (.62 and .75mM, *p*NPP and casein, respectively).

In this study, the pAPase bound with the carbodiimide procedure had measurable activity

in removing phosphate groups from whole casein. Dephosphorylation as low as 4% on whole casein showed a faint DP β -casein zone upon urea-PAGE. When 15% of total phosphate groups were removed, three DP β -casein and three to four faint DP α_{s1} -casein zones were observed. Ohmiya et al. (14) incubated a 10% dispersion of skim milk with glutaraldehyde-immobilized wheat germ and sweet potato APase at 30°C, pH 6.4 for 9 h. The PAGE

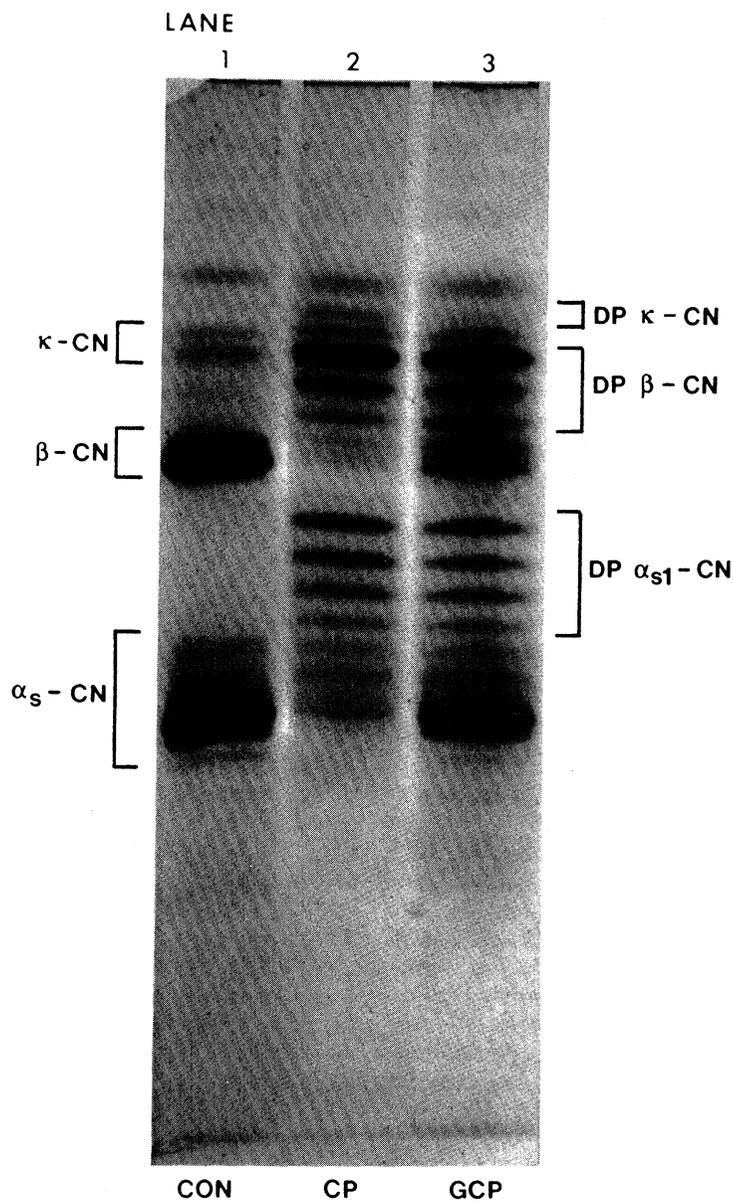


Figure 4. Urea-PAGE (7% acrylamide gel) profile of native (lane 1, CON) and partially dephosphorylated (DP) bovine whole casein (CN) after 24-h batch incubation with potato acid phosphatase immobilized on either controlled pore glass beads (CP) (lane 2) or glycerol-coated controlled pore glass beads (GCP) (lane 3). Protein concentrations were approximately 150 μ g/slot.

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patterns revealed negligible shifts in mobilities of major caseins, indicating minimal dephosphorylation had occurred.

As reported herein (Table 2), specific activity of the free enzyme decreased from the 15- to 20-min rate until 4 h when casein was the substrate. Because dialysis was not incorporated in the 4-h study, phosphate inhibition likely contributed to the decrease (1, 5). The specific activity rate for the immobilized enzymes decreased from 4 h to 18 or 24 h. Although the rate was steady by 18 h, the 18 and 24-h incubation experiments included dialysis to remove inhibitory phosphate as it was released. The decrease in activity, despite the removal of phosphates through dialysis, suggested that additional factors influenced the rate of the dephosphorylation of casein, including alterations of the enzyme, i.e., inactivation or clogging of enzyme-bound support. It could also be due to the substrate, which, as each phosphate group is removed, requires higher energy to release the remaining phosphate groups because of conformational changes in the protein. A very small amount of residual phosphate has always been reported for casein, even after extensive dephosphorylation (2, 3, 4, 5, 29).

Urea-Polyacrylamide Gel Electrophoresis

Urea-PAGE patterns (Figures 3 and 4) of the modified casein illustrates the slower mobility of DP α_s -casein and β -casein fractions referred to in other studies (2, 4, 5, 13, 14, 21, 23, 26, 29). Native β -casein had five phosphate groups and shows one major and one minor zone. Three DP β -casein zones are visible with the maximally dephosphorylated zone located in the native κ -casein region. Because κ -casein had only one phosphate per molecule, the resulting DP κ -casein zone was difficult to identify, especially with the DP β -casein band overlapping the area. The α_{s1} -casein had eight to nine phosphates to be removed and showed a major and minor zone on urea-PAGE. Partial dephosphorylation of α_s -casein resulted in four zones behind native α_{s2} -casein with the maximum DP zone migrating ahead of native β -casein. Native α_{s2} -casein appeared as two minor zones behind the α_{s1} -casein zones, but when phosphate groups were removed, the DP

zones were mixed with DP α_{s1} -casein, DP β -casein, and β -casein. Maximum DP α_{s2} -casein had been reported to migrate slower than native β -casein (2).

Long-Term Incubation

Three milligrams of whole casein per milliliter used in this study contained approximately .86 mM phosphate. Dialysis was incorporated into the long-term incubations to remove inhibitory phosphates and to facilitate maximum dephosphorylation. The 24-h batch incubation was contained within a single dialysis membrane submerged in an 18-L dialysis tank. This system resulted in 35% DP caseins for the CP. Inadequate mixing of complex with the substrate may be responsible for the low DP casein yields. A second reaction system was designed with continuous reverse flow through a column and attached dialysis membrane. This system resulted in increased yields of DP casein, which turned out to be proportional to the increase in CP:substrate or GCP:substrate ratio rather than to improved efficiency.

CONCLUSIONS

Potato APase was successfully immobilized on succinamidopropyl porous glass beads with measurable activity toward both *p*NPP and whole casein substrates. Porous glass beads bound approximately .5 mg of enzyme/g of support. Optimum pH ranges were not changed from pH 5.5 for *p*NPP and pH 6.0 to 6.5 for casein. Immobilized enzyme specific activities for *p*NPP substrate were less than 15% of free enzyme activity. Specific activity of the bound enzyme for whole casein, although less than for *p*NPP, resulted in observable dephosphorylation. The activity of the enzyme toward casein decreased with time but stabilized by 18 h. Maximum dephosphorylation (70%) was obtained with 2.6 mg CP/mg casein in buffered solution at pH 6.5 and incubated at 37° for at least 18 h.

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