Purification of Pregastric Lipases of Caprine Origin[1]

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ABSTRACT: Pregastric lipases from kid (KPGL) and goat (GPGL) were purified from the commercial extracts by different chromatographic procedures. The total recovery of activity for both purification methods was *ca.* 10%, and the specific activities of KPGL and GPGL were 533 and 546 U/mg, respectively, at pH 6.5, 35°C for tributyrylglycerol (TBG) as substrate in a casein/lecithin emulsion. The purification factors were 130- and 76-fold for the goat and kid lipases, respectively. The purified lipases from kid and goat showed the same 50 kDa protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and an identical sequence for the first 11 amino acids. The optimal pH for the lipases was within the pH range 6–7, with maximal activity at pH 6.5. The stability of the purified lipases was decreased dramatically at pH > 6.5, but was enhanced by the addition of albumin.

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KEY WORDS: Albumin, enzyme purification, enzyme stability, goat, inactivation rate constant, kid, molecular weight, 4-nitrophenylacetate, pregastric lipase, tributyrylglycerol.

Pregastric (lingual) lipase (E.C. 3.1.1.3, triacylglycerol acylhydrolase) (PGL) is secreted from the serous glands, which are located on the dorsal, proximal side of the tongue of all mammals. The enzyme plays a major role in fat digestion, especially for newborn or premature infants (1,2). From the biotechnological point of view, especially with respect to utilization of these enzymes in fat processing, the lipases from ruminants (lamb, kid, and calf) have long been used for development of flavor in cheese and other dairy products, and each type of PGL gives rise to its own characteristic flavor. Kid PGL is especially unique in its ability to produce a strong picante flavor in Italian-style cheeses. However, the lipase is extracted from the tongues of 14 d-old-kids, which are not available in some countries, and so, substitution of the kid lipase by that from the adult goat or other mammal has become of industrial interest.

Early investigations on the pregastric enzymes have been based on the crude or partially purified enzymes (3), and the structure and mechanism of their catalyzed reactions have not been discussed. Recently, however, the purification of the lingual lipases or PGL from calf (4–6), lamb (7,8), rat (9,10), and human (10–12) has been achieved, and the lipases reveal similar characteristics, even when extracted from different animals. Determination of the molecular weight of these PGL shows them to be glycoproteins with a high ratio of carbohydrate content and with molecular weights in the range of 43–51 kDa and highly homologous amino acid sequences.

Different purification methods have been attempted, depending on the source of the enzyme, but most have suffered from a low recovery of lipase activity. In this study, two separate purification methods have been developed for kid and goat pregastric extracts, and high yields and purification factors have been achieved. Some characteristics of the purified lipases are discussed.

MATERIALS AND METHODS

Materials. Freeze-dried PGL extract was a gift from New Zealand Rennet Co. Ltd. (Eltham, New Zealand), and the kid pregastric extract was from Marschall Products, Rhône-Poulenc (Madison, WI). Sepharose-Q fast-flow and Sephacryl S-100 were purchased from Pharmacia LKB (Uppsala, Sweden), and Affi-gel Blue, ceramic hydroxyapatite (Type I), the protein assay kit, and electrophoresis reagents were Bio-Rad (Richmond, CA) products. Bovine serum albumin (BSA), 1,3-bis[tris(hydroxymethyl)-methylamino]propane (BTP), SigmaMarker, tributyrylglycerol (TBG), L-α-lecithin and 4-nitrophenylacetate (PNPA) were from Sigma (St. Louis, MO). *N*-Morpholinoethanesulfonic acid (Mes) was a Serva product (Heidelberg, Germany). Tris(hydroxymethyl)aminomethane (Tris) was from United States Biochemical Corp. (Cleveland, OH). Dithiothreitol was from BDH (Poole, England).

Determination of protein concentration. Protein concentration was determined by the method of Bradford (13) with the Bio-Rad protein assay kit and BSA as standard.

Esterase activity. Esterase activity was measured spectrophotometrically (H-P 8452A spectrophotometer, Hewlett-Packard Co., Int., Palo Alto, CA) at pH 6.5, 35°C with PNPA as substrate (14). The reaction vessel contained 3 mL 50 mM BTP buffer (pH 6.5), 50 μL 60 mM PNPA, and 50 μL enzyme solution (variable concentration), and the released 4-nitrophenolate anion was monitored at 400 nm, 35°C.

Lipase activity. The lipase activity of the enzyme was measured titrimetrically (Mettler DL21 autotitrator; Mettler In-

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struments AG, Greifensee, Switzerland) at pH 6.5, 35°C (15). TBG (100 mg) was added to 40 mL lecithin solution (1%, w/w) and sonicated until it became monodisperse. Released butyric acid was titrated with 10 mM NaOH. The specific activity (U/mg) of the enzyme was defined as the amount of lipase catalyzing the release of 1 μmol butyric acid per minute.

Purification of goat PGL (GPGL). The crude enzyme extract (1 g) was dissolved in 50 mM Tris-HCl buffer, pH 8. After centrifugation for 15 min at 10,000 rpm, the supernatant was loaded onto a Sepharose-Q column (1.6 × 20 cm) at a flow rate of 1.0 mL/min. The column was washed with 5 column vol of 50 mM Tris-HCl, pH 8, and then equilibrated with another 5 column vol of 50 mM Mes buffer, pH 5.6, before elution of the bound material with a linear gradient of 0–0.8 M NaCl in 50 mM Mes buffer, pH 5.6. Eluents were monitored at 280 nm. The collected lipase fractions were then dialyzed against 20 mM Tris, pH 8. The dialysate was added to 15 mL Affi-gel Blue, equilibrated with 20 mM Tris-HCl, pH 8.0, and the mixture was incubated at 4°C for 2 h in a horizontal shaker. The nonbound fraction was concentrated by adding an equal volume of 4 M $(NH_4)_2SO_4$, and after stirring at 4°C for 1 h, the precipitate was collected.

This precipitate was redissolved in Milli-Q water and extensively dialyzed against 50 mM Tris-HCl, which contained 0.1 M NaCl and 0.2 mM dithiothreitol. The dialyzed enzyme solution was then loaded onto a Sephacryl S-100 column (1 × 200 cm) at a flow rate of 1.0 mL/min. The eluted active fractions were collected, concentrated, and desalted in an Amicon Centricon-10 filter unit (Amicon Inc., Beverly, MA). No significant loss of activity was observed for the purified lipase after keeping it for 2 mon at 4°C in 10 mM Mes, pH 6.5 with 3 mM NaN_3.

Purification of kid PGL (KPGL). The crude kid extract (2 g) was dissolved in 50 mM Tris-HCl, pH 8, centrifuged, and applied onto a Sepharose-Q ion-exchange column by the method described above. The collected lipase fractions were dialyzed against 10 mM potassium phosphate buffer, pH 6.7, and the dialyzed enzyme solution was loaded onto a column of ceramic hydroxyapatite (1 × 16 cm). The bound material was washed by 2 column vol of 10 mM phosphate buffer and eluted with a linear phosphate gradient (10–200 mM) at a flow rate of 0.6 mL/min. Eluents were monitored at 280 nm. The fractions with lipase activity were then concentrated and purified by gel filtration chromatography as described above.

Electrophoresis. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for determining enzyme purity (16). Each sample was subjected to a 10% gel and stained by Coomassie blue. Sigma-marker was used as standard.

N-*Terminal sequence analysis.* The protein band on the SDS gel that was identified as lipase was transferred to ProBlott™, and the *N*-terminal sequence was determined by automatic protein sequencing (Procise 492; Applied Biosystems, Foster, LA).

Enzyme stability. For determination of enzyme stability against pH, an equal volume of 100 mM phosphate buffer (variable pH) was added to the purified lipase solution. The effect of BSA on lipase stability was performed by premixing equal volumes of enzyme solution and BSA in 100 mM Mes buffer, pH 6.5. The diluted enzyme solution was then incubated at 50°C, and the activity against TBG at pH 6.5, 35°C was measured sequentially until 90% of the original activity had been lost. The loss of lipase activity followed exponential decay, and the inactivation rate constant was calculated.

RESULTS AND DISCUSSION

Chromatography procedures used for purification of pregastric enzymes have been based on three different but general steps: (i) anion-exchange chromatography, to partition and concentrate the active enzyme fraction and remove nonprotein contaminants; (ii) affinity chromatography, with several different affinity resins [e.g., Econo-Pac blue, lectil-lectin Sepharose, or hydroxyapatite has each been used for purification of lamb (7,8) and calf PGL (6)]; (iii) gel filtration, to isolate the final active component. Timmermans *et al.* (5) have recently developed a two-step purification procedure and obtained calf PGL of high purity with 14% total recovery of activity and a 13-fold purification factor. However, no single procedure is suitable for purification of PGL from a range of mammals. The variation of protein composition, caused by the use of different commercial methods to extract the crude protein and the presence of tissue in these crude extracts, causes difficulties in subsequent purifications. Although we demonstrate here two purification methods for the two commercial enzyme extracts from goat and kid, the purified lipases show identical characteristics with respect to molecular weight and *N*-terminal sequence.

For the ion-exchange chromatography, a high pH value for the loading buffer was chosen because the binding capacity of the enzyme at pH 8 is almost double that at pH 5.5, with over 90% of the enzyme activity being bound onto the resin. Lowering of the pH to 5.6 then caused dissociation of a major contaminant, creatine kinase, from the resin without loss of lipase activity.

Two different assay methods, hydrolysis of PNPA for both esterase and lipase activities and hydrolysis of TBG for lipase activity only, were used to monitor the enzyme activities during purification. Figure 1A and 1B show elution profiles for the ion-exchange chromatography of the goat and kid extracts, respectively. For the goat enzyme extract, the esterase component was eluted within the gradient range of 0.1–0.2 M NaCl, and lipase activity was found with 0.4–0.6 M NaCl. The resolution of the two active components was decreased when the gradient elution was performed at pH > 7. A sixfold purification factor was found for the lipase at this stage.

For the kid enzyme extract, both esterase and lipase activity appeared to be coeluted over the range 0.4–0.6 M NaCl. The use of a PNPA assay alone is not necessarily indicative of the presence of lipase because we have shown that both pregastric esterase and PGL are active against PNPA (14). However, a TBG assay does distinguish the lipase component

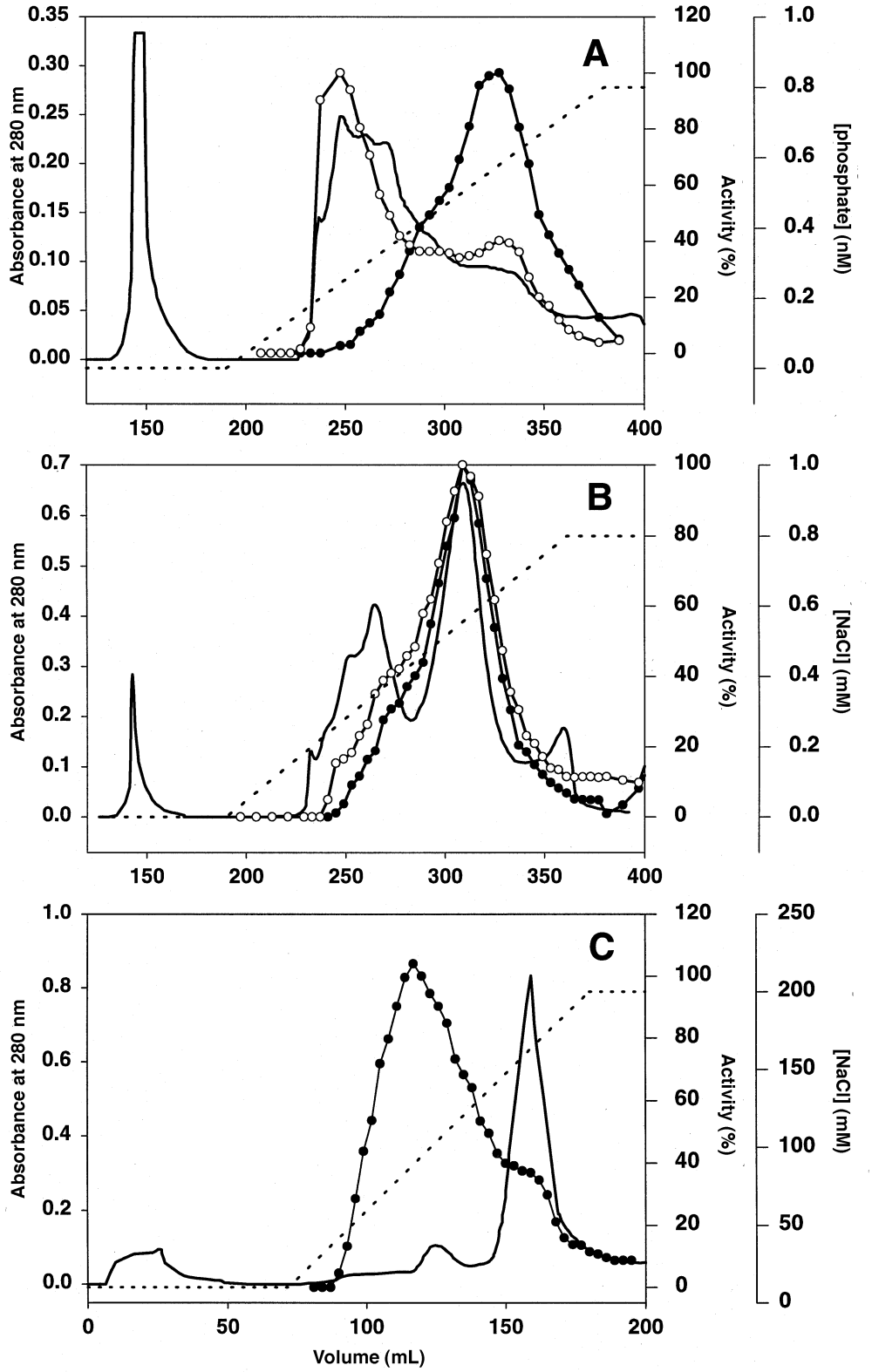

FIG. 1. Sepharose-Q chromatography of goat (A) and kid (B) pregastric extract and hydroxyapatite chromatography (C) of the fraction that contained lipase activity (NaCl 0.4–0.6 M) from separation (B). Eluents were monitored at 280 nm (—). Lipase activity was measured against tributylglycerol (TBG) (●—●), and esterase activity was measured against 4-nitrophenylacetate (PNPA) (○—○). Chromatography was performed by a linear NaCl or phosphate gradient (·····).

TABLE 1
Flowsheet for the Purification of Goat and Kid Pregastric Lipases

Purification step	Total activity (units)	Specific activity (units/mg)	Total protein (mg)	Activity recovery (%)	Purification factor
Crude goat enzyme extract (1 g)	2272	4.2	540.6	100%	1
Sepharose-Q chromatography	1181	23.5	61.6	52%	5.6
Affi-gel Blue	759	56.7	13.4	33%	13.5
Sephacryl S-100	233	546.2	0.4	10%	130
Crude kid enzyme extract (2 g)	4533	7.0	646.1	100%	1
Sepharose-Q chromatography	2901	23.5	61.6	64%	2.2
Ceramic hydroxyapatite	1632	224.5	7.3	36%	32
Sephacryl S-100	635	533.2	1.2	14%	76

from the esterase. The esterase activity in the goat extract may be due to contamination by serum in the original commercial extract. Moreover, the presence of an esterase component does not affect the degree of hydrolysis of a lipid because identical free fatty acid profiles have been obtained for hydrolysis of milk fat catalyzed by both crude goat extract and purified GPGL (17).

Figure 1C shows the elution profile for hydroxyapatite chromatography of the fractions obtained from the 0.4–0.6 M NaCl gradient elution from the Sepharose-Q chromatography of the kid enzyme extract. The fraction with the lipase activity was eluted within the gradient range of 50–100 mM phosphate, and a 15-fold further purification was achieved by this step. Hydroxyapatite chromatography was for purification of PGL in terms of capacity and cost, and it has previously been found to be a crucial step for purification of lamb PGL (8). Absorption of GPGL on the resin was poor compared with KPGL, possibly owing to a protein–protein interaction, which reduces the affinity of PGL to the resin. Further work is under way to simplify the purification procedure and increase the quantity of purified PGL.

Table 1 shows the flowsheet for purification of KPGL and GPGL. The final purification factors were 130 for GPGL and 76 for KPGL, with a total recovery of activity of 10 and 14% for GPGL and KPGL, respectively. The specific activities for GPGL and KPGL were 546 and 533 U/mg, respectively, at pH 6.5, 35°C. The activities of KPGL and GPGL are of the same order as calf PGL [905 U/mg (4)] and lamb PGL [260 U/mg (8)], especially when one considers that these literature values are reported for determinations made under different conditions.

The results of SDS-PAGE analysis at various purification steps for GPGL and KPGL are shown in Figure 2. The purified GPGL and KPGL are shown as a single protein band with a molecular weight of 50 kDa (Lane 4 and Lane 5), a value identical to those of calf (4) and lamb (8) PGL. Table 2 compares the *N*-terminal sequences of KPGL and GPGL with those for calf and lamb PGL. The first 11 amino acid residues for KPGL and GPGL are identical to those for lamb PGL, but differ from calf PGL at the eighth base. In calf PGL, this base is lysine (K), and in the other PGL, it is glutamic acid (E).

Both KPGL and GPGL were active over a wide range of pH values. Figure 3 shows pH profiles for the catalyzed activity against TBG of purified goat and kid PGL in 1% (w/w) lecithin emulsion. Optimal activity was within the pH range 6–7, with maximal activity being observed at pH 6.5. Other PGL have shown optimal pH ranges of 5–6 [purified lamb PGL against TBG in gum arabic emulsion (8)], and 4.5–6.5 [calf PGL against olive oil in BSA emulsion (18)].

Purified GPGL was stable within the pH range 4.5–6.5. Figure 4 shows the pH profile for the inactivation rate constant (k_i) of GPGL at 50°C. At higher pH values, GPGL was unstable, as evidenced by a sudden increase in k_I at pH >6.5. Values of k_I at pH values below 4.5 could not be measured because the GPGL precipitated and suffered total inactivation.

Purified forms of the PGL are less stable (4,7,8) and easily denatured at a lipid/water interface. Addition of BSA (up to

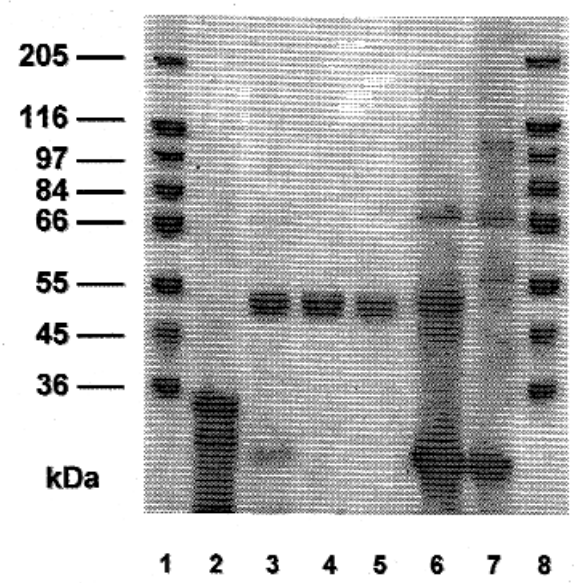

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) patterns of kid pregastric lipases (KPGL) and goat pregastric lipases (GPGL) at various stages of purification. Lanes 1 and 8: molecular mass markers; lane 2: pooled active fractions from Sepharose-Q for GPGL; lane 3: unbound GPGL solution from Affi-gel Blue chromatography; lane 4: purified GPGL after Sephacryl S-100 chromatography; Lanes 5, 6, 7; pooled fractions of KPGL after Sephacryl S-100, hydroxyapatite and Sepharose-Q chromatography, respectively.

TABLE 2
***N*-Terminal Amino Acid Sequences for PGL from Kid, Goat, Calf (3), and Lamb (6)**

PGL	Acid sequence										
Kid	F	L	G	K	I	A	E	N	P	E	A
Goat	F	L	G	K	I	A	E	N	P	E	A
Lamb	F	L	G	K	I	A	E	N	P	E	A
Calf[a]	F	L	G	K	I	A	K	N	P	E	A

[a]The amino acid sequence of calf pregastric lipase (PGL) was deduced from the cloned DNA.

25 μM) stabilized GPGL (Fig. 5), implying that the BSA–lipase interaction plays an important role in stabilizing the enzyme.

To our knowledge, we are the first to report the purification of KPGL and GPGL. In contrast to a previous comparison, made between calves and adult steers (19), caprine enzyme activity does not vanish as the young kid grows, even though the adult goat does not consume significant amounts of lipids. The kid and goat lipases are similar to each other and to lamb PGL in terms of their molecular weight, amino acid sequence, activity, and substrate selectivity (17). Thus, utilizing the PGL from either adult goat (or lamb) should be alternatives to the valuable KPGL in biotechnological processes.

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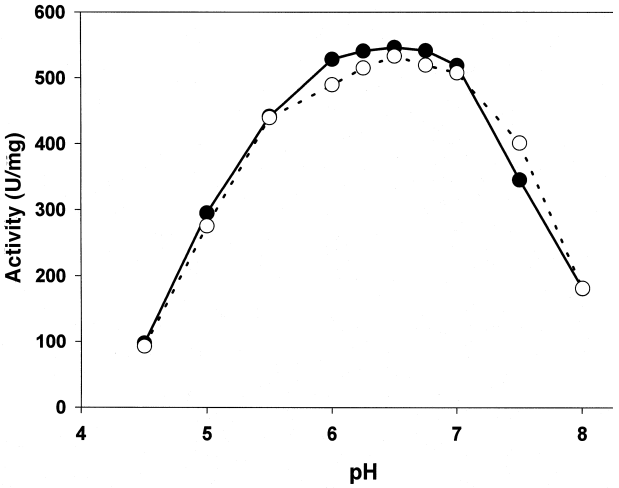

FIG. 3. Effect of pH on hydrolysis of TBG catalyzed by purified GPGL (●—●) and KPGL (○····○) in 1% (w/w) lecithin emulsion. See Figures 1 and 2 for abbreviations.

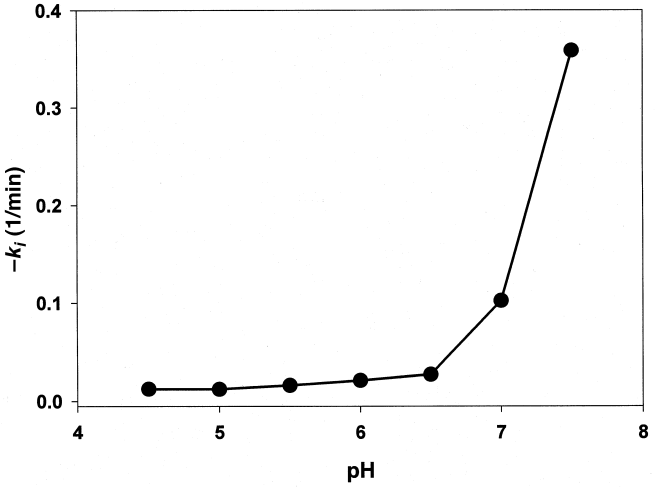

FIG. 4. Effect of pH on the inactivation rate constant for KPGL at 50°C. See Figure 2 for abbreviation.

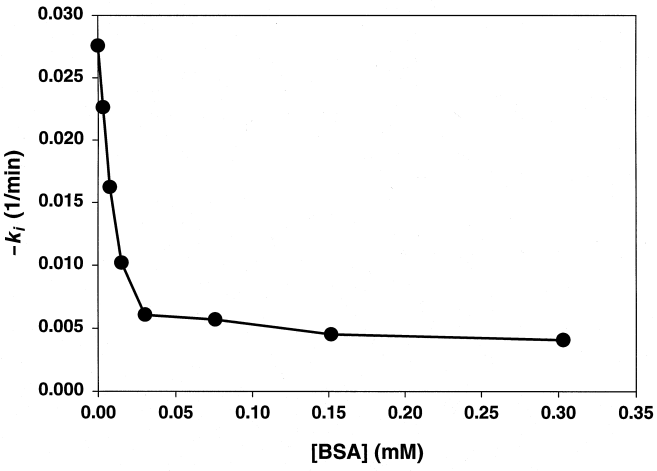

FIG. 5. Effect of bovine serum albumin (BSA) concentration on the inactivation rate constant for KPGL at pH 6.5, 50°C.

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