Stability of Acyl Derivatives of Insulin in the Small Intestine: Relative Importance of Insulin Association Characteristics in Aqueous Solution

Hiroyuki Asada,[1,2] Takayuki Douen,[1] Yoshiko Mizokoshi,[1] Takuya Fujita,[1] Masahiro Murakami,[1] Akira Yamamoto,[1] and Shozo Muranishi[1]

Received October 4, 1993; accepted April 5, 1994

The stability of insulin and its acyl derivatives in the small intestine was examined *in vitro*. When these compounds were incubated in small intestinal fluid at 37°C, proteolysis of monoacyl insulins was reduced by increasing the carbon number of the fatty acid attached to Phe-B_1 of the insulin molecule. In contrast, Phe-B_1 and Lys-B_{29} diacylated insulins were more susceptible to hydrolysis than native insulin. Similar results were obtained using homogenates of the small intestinal mucosa, although the extent of the contribution of acylation to insulin degradation differed. The mechanism of the accelerated insulin proteolysis by diacylation was studied by circular dichroism (CD). The negative maxima at 270 nm in the CD spectra were attenuated for the diacyl derivatives, indicating that insulin association was inhibited by diacylation. Therefore, the increased proportion of monomers available for insulin proteolysis represents a main factor that makes diacyl derivatives unstable.

KEY WORDS: insulin; chemical modification; fatty acids; intestinal stability; association; circular dichroism.

INTRODUCTION

The poor absorption characteristics of insulin after oral administration is partly explained by the rapid degradation of this peptide by the pancreatic digestive enzymes present in the intestinal lumen, such as trypsin and α-chymotrypsin, or brush border enzymes of the intestinal mucosa (1–3). To improve the stability of insulin, thereby increasing its absorption from the gastrointestinal tract, various protease inhibitors have been used for several years (4,5). Further, dosage form modification with liposome and emulsion is also an important means of improving the intestinal stability and absorption of insulin (6,7). However, few studies have examined the improvement of stability of insulin by chemical modification.

We synthesized lipophilic derivatives of thyrotropin-releasing hormone (TRH), tetragastrin and insulin (8–10). Yamada *et al.* reported that Lau-TRH, a novel derivative of TRH with chemically attached lauric acid at the N-terminal pyroglutamyl group, was much more stable than TRH in rat plasma (11). The acyl derivatives of tetragastrin are better absorbed from the large intestine than native tetragastrin, which may be partly due to the higher stability of these derivatives in the large intestine compared with the parent (9). Among the acyl derivatives of insulin, palmitoyl insulins are more stable than native insulin in mucosal tissue homogenates of the large intestine (12). These results suggested that chemical modification of peptides with various fatty acids reduces their degradation, resulting in increased absorption from the gastrointestinal tract. However, it has not been examined in detail to what extent the chain length and the numbers of fatty acids attached to insulin influence its stability in the gastrointestinal tract.

In this study, we synthesized lipophilic derivatives of insulin by chemical modification with various fatty acids and investigated their stability in both small intestinal fluid and homogenates of the small intestinal mucosae. In addition, to clarify the mechanisms of the alteration of insulin stability by acylation, the association characteristics of these derivatives were also investigated by circular dichroism.

MATERIALS AND METHODS

Materials

Crystalline bovine insulin (25.7 IU/mg) was purchased from Sigma Chemical Company (St. Louis, MO, USA), and palmitic acid, lauric acid, caproic anhydride, N,N-dimethylformamide (DMF) and N-hydroxysuccinimide were purchased from Nacalai Tesque Co. (Kyoto, Japan). p-Methoxybenzoxycarbonyl azide (*p*MZ-azide) was obtained from Watanabe Chemical Industry (Hiroshima, Japan). Trifluoroacetic acid (TFA) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of reagent grade.

Synthesis of Acyl Derivatives of Insulin

Acyl derivatives of insulin were synthesized as described previously with a slight modification (10). In brief, insulin (60 mg) was reacted with five volumes (mol) of *p*MZ-azide (11 mg), the protecting reagent for the amino group, since glycine-A_1 is essential for insulin activity (13,14). A_1-mono-*p*MZ-insulin and A_1, B_{29}-di-*p*MZ-insulin were obtained by the above reaction. B_1-monoacyl insulin and B_1, B_{29}-diacyl insulin were produced by the coupling of A_1, B_{29}-di-*p*MZ- and A_1-mono-*p*MZ-modified insulin with a fatty acid, respectively, followed by treatment with TFA in the presence of anisole in order to remove the *p*MZ group. To form insulin containing palmitic and lauric acids, *p*MZ-modified insulin was coupled with 50 volumes (mol) of their N-hydroxysuccinimide esters, Pal-Osu and Lau-Osu, respectively. To couple with caproic acid, *p*MZ-modified insulin was coupled with 1.5 volumes of caproic anhydride, because it is difficult to crystallize Cap-Osu. The final purification was achieved by preparative HPLC. The structure of the acyl derivatives synthesized is shown in Fig. 1. The acylation of the final products was confirmed by amino acid analysis of deaminated derivatives. Lipophilic indexes for insulin and its acyl derivatives, were calculated from the retention times of these compounds as follows:

$$\text{Lipophilic Index} = \log (t_R - t_0)/t_0$$

[1] Department of Biopharmaceutics, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607 (Japan)

[2] To whom correspondence should be addressed.

0724-8741/94/0800-1115$07.00/0

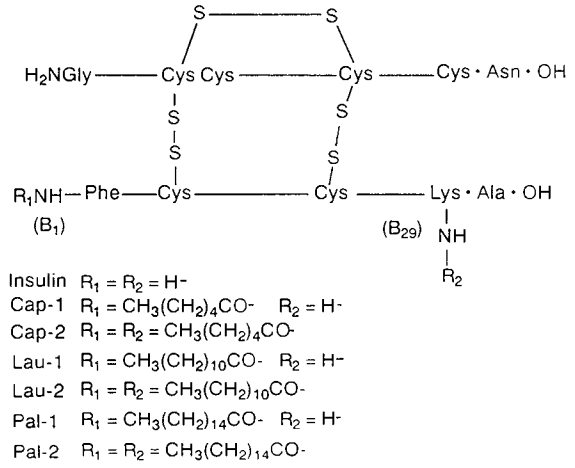

Fig. 1. Structure of insulin and its acyl derivatives studied here.

where t_R and t_0 are the retention times of each compound and solvent, respectively.

Preparation of Rat Small Intestinal Fluid

The small intestinal fluid was collected from rat by the modified method described previously (15). Three male Wistar albino rats, weighing 250–300 g, (Japan SLC, Inc., Hamamatsu, Japan) were anesthetized with sodium pentobarbital (30 mg/kg) intraperitoneally. Small intestinal contents were washed out two times with 10 ml of distilled water. This efflux was extracted five times with two volumes of methylene chloride to remove lipids that may interfere with the HPLC analysis of insulin and its acyl derivatives. This extract was diluted with distilled water to yield a protein concentration of 1 mg/ml and used as small intestinal fluid.

Preparation of Rat Small Intestinal Mucosal Homogenates

Mucosal homogenates of rat small intestine were prepared by the modified method of Yamamoto *et al.* (16) as reported previously. The small intestine removed from rat was divided into three parts. First, 3 cm of the top of the small intestine was removed and the duodenum segment was obtained from the next 10 cm portion. The ileum was obtained from the final 10 cm portion of the intestine. The residual intestine was designated as the jejunum. The mucosal tissue was scraped with plate glass and homogenized in 3 volumes of isotonic phosphate buffer (PBS, pH 7.4) using a POLYTRON homogenizer (Kinematica, GmbH, Switzerland). The homogenate was separated by centrifugation at 5600 × g at 4°C for 10 min. The supernatant was diluted with PBS to a protein concentration of 10 mg/ml.

Degradation of Insulin and Its Acyl Derivatives in the Small Intestinal Fluid and Homogenates of Small Intestinal Mucosa

The degradation of insulin and its acyl derivatives was studied by incubating 100 μl of the small intestinal fluid with 400 μl of insulin or its acyl derivatives. Test solutions were prepared by dissolving each compound first in DMF, a solubilizing agent, followed by dilution with PBS to a final insulin concentration of 50 μM in 4% DMF. Similarly, 100 μl of a supernatant of tissue homogenates was incubated with 400 μl of 0.1 mM test solution. At predetermined times up until 180 min, 50 μl of samples were withdrawn from the incubation mixture. After adding 50% acetic acid to terminate the reaction, the sample was immediately separated by centrifugation at 2300 × g at 4°C for 5 min. The remaining insulin and its acyl derivatives in the supernatant were determined by HPLC.

Table I. A Comparison of Lipophilicity of Insulin and Its Acyl Derivatives

	Tr	Lipophilic Index	Lipophilicity
Native Insulin	15.8	0.673	1.000
Cap-1	16.3	0.687	1.021
Cap-2	17.6	0.732	1.088
Lau-1	20.3	0.812	1.207
Pal-1	23.8	0.896	1.331
Lau-2	24.9	0.919	1.366
Pal-2	34.4	1.092	1.623

Lipophilic Index = $\log (t_R - t_0/t_0)$ (t_0: Retention time of solvent).
HPLC Condition:
Column: YMC-AM302 (ODS) (4.6 × 150 mm).
Eluant: 0.1% TFA (A) with CH_3 CN (B) at a gradient Concentration 20 to 100% in 48 min.

HPLC Assay of Insulin and Its Acyl Derivatives

Insulin and its acyl derivatives were assayed by reversed phase HPLC on Vydac protein and peptide C_{18} column (250 × 4.6 mm, 5 μm). The HPLC consisted of a Waters LC module 1 and a Shimadzu model C-R4A integrator. The mobile phase was a mixture of 0.1% TFA (mobile phase A) and acetonitrile (mobile phase B). To assay native insulin, Cap-1, Cap-2, and Lau-1, the gradient system was programmed by increasing the proportion of mobile phase B

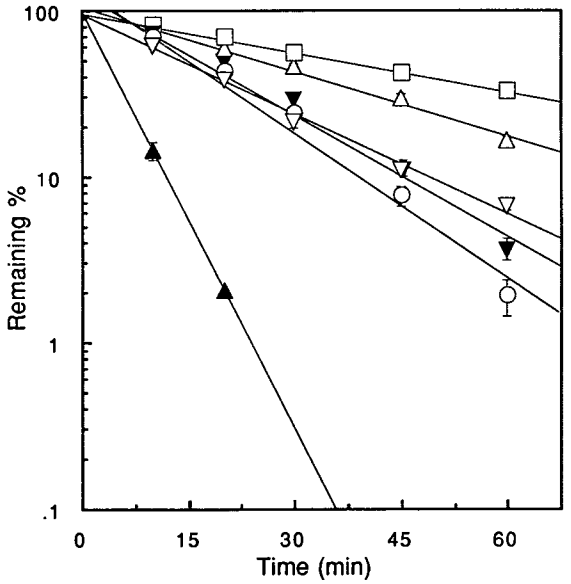

Fig. 2. Semilogarithmic plots of the proteolysis of insulin and its acyl derivatives in the small intestinal fluid at 37°C. Key: (○), Native insulin; (▽), Cap-1; (▼), Cap-2; (△), Lau-1; (▲), Lau-2; (□), Pal-1.

from 20 to 80% within 30 min. For Lau-2 and Pal-1, that was increased linearly from 20 to 100% within 30 min. The mobile gradient phase was run at a flow rate of 1 ml/min. The ultraviolet detector was set at 210 nm.

Circular Dichroism Studies

A circular dichroism (CD) spectropolarimeter (JASCO Model J 500, Japan Spectroscopic Co., Tokyo) was utilized to scan from 300 to 250 nm at a rate of 25 nm/min at 25°C. Optimal resolution of the spectra was obtained using a 1 cm-pathlength quartz cuvette. The molar ellipticity was calculated from the equation:

$$[\theta]_\lambda = \frac{\theta_\lambda}{C \times 1} \times 100$$

where θ_λ is the observed ellipticity at wavelength λ in degree, C is the molar insulin concentration, and 1 is the pathlength in centimeters.

Statistical Data Analysis

Results are expressed as the means ± S.E. Statistical data analysis were performed using the *t* test with $p < 0.05$ as the minimal level of significance.

RESULTS

Lipophilicity of Insulin and Its Acyl Derivatives

Table I shows the lipophilicity of insulin and its acyl derivatives. The lipophilic indexes of these acyl derivatives were higher than that of native insulin, indicating that these derivatives were more lipophilic than native insulin. In addition, the lipophilicity of these compounds increased with increasing the carbon numbers of the fatty acid and/or the number of fatty acids that were chemically attached to the native insulin.

Stability of Insulin and Its Acyl Derivatives in the Small Intestinal Fluid

Figure 2 shows the time course of proteolysis of insulin and its acyl derivatives by incubating with the small intestinal fluid. Native insulin was rapidly degraded in this fluid and less than 10% of the insulin remained at 45 min. The proteolysis of monoacyl derivatives in the small intestinal fluid was reduced with an increase in the carbon number of the fatty acid which was attached to insulin. In contrast, diacyl derivatives were more susceptible to hydrolysis in the small intestinal fluid than native insulin. Table II shows the half-lives for the proteolysis of insulin and its acyl derivatives in the small intestinal fluid which were calculated from the first order rate constants. Although the half-lives for the proteolysis of Cap-1 and Cap-2 were similar to that of native insulin, that of Pal-1 significantly increased 3.5-fold under the above conditions. The half-life for proteolysis of Lau-2, however, significantly decreased 0.35-fold.

Table II. The Half-Lives for Proteolysis of Insulin and Its Acyl Derivatives in Small Intestinal Fluid

	$T_{1/2}$ (min)	Ratio
Native Insulin	10.4 ± 0.6	1.00
Cap-1	14.9 ± 0.3*	1.43
Lau-1	23.5 ± 1.1*	2.26
Pal-1	36.5 ± 0.9**	3.51
Cap-2	10.6 ± 0.5	1.02
Lau-2	3.6 ± 0.02*	0.35

Results are expressed as means ± S.E. of 3 experiments.
* $P < 0.01$, ** $P < 0.001$, significantly different from the half-life of proteolysis of native insulin.

Stability of Insulin and Its Acyl Derivatives in Homogenates of the Various Intestinal Mucosae

Table III shows the half-lives for the proteolysis of insulin and its acyl derivatives in the homogenates of various intestinal mucosae. For native insulin, the rank order of proteolytic rate among various intestinal mucosal homogenates was as follows; (the small intestinal fluid ≫) jejunum > duodenum > ileum (≫ colon, 10 cm of the first large intestine). The half-life for insulin hydrolysis in the colonic mucosal homogenate was calculated to be 330 ± 40 min (mean ± S.E.), indicating that insulin is more stable in the large intestine than in the small intestine.

The proteolysis of monoacyl insulins in homogenates of each small intestinal site was reduced with an increase in the carbon number of the fatty acid. Compared with native insulin, Pal-1 was 3.4- and 2.4-fold more stable in the duodenal and ileal mucosa, respectively, and significantly 1.7-fold

Table III. The Half-Lives for Proteolysis of Insulin and Its Acyl Derivatives in the Homogenates of Various Intestinal Mucosae

	Duodenum		Jejunum		Ileum	
	$T_{1/2}$ (min)	Ratio	$T_{1/2}$ (min)	Ratio	$T_{1/2}$ (min)	Ratio
Native Insulin	58 ± 2	1.0	21 ± 2	1.0	63 ± 1	1.0
Cap-1	72 ± 6	1.2	29 ± 1*	1.4	83 ± 4*	1.3
Cap-2	68 ± 4	1.2	28 ± 4	1.4	26 ± 0***	0.4
Lau-1	143 ± 5***	2.5	20 ± 4	1.0	108 ± 9*	1.7
Lau-2	30 ± 2***	0.5	7 ± 1**	0.3	21 ± 2***	0.3
Pal-1	200 ± 16**	3.4	35 ± 4*	1.7	153 ± 6***	2.4

Results are expressed as means ± S.E. of 3 experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from the half-life of proteolysis of native insulin.

more stable even in the jejunal mucosa, where insulin was most susceptible to hydrolysis.

Association Characteristics of Insulin and Its Acyl Derivatives

In order to clarify the mechanisms for the acceleration of insulin proteolysis by diacylation, the effect of acylation on insulin association was investigated by circular dichroism (CD). As shown in Fig. 3, on the CD spectra of both monoacyl and diacyl derivatives, negative maxima at about 270 nm was attenuated. The attenuation of negative maxima on the CD spectra of Lau-2 and Pal-2 was remarkable. The minimum molar ellipticities at about 270 nm are summarized in Table IV and the relationship between these values and lipophilicity are shown for monoacyl and diacyl derivatives, respectively, in Fig. 4. We confirmed that the effect of acylation on insulin association was also dependent on the chain length of the fatty acid attached to insulin.

DISCUSSION

Native insulin was rapidly degraded in the small intestinal fluid and homogenates of various small intestinal mucosae, as reported previously (17), and this enzymatic barrier is considered a main factor limiting insulin absorption from the small intestine (18). On the other hand, the monoacyl derivatives were more stable than native insulin in the small intestine, whereas insulin proteolysis was accelerated by diacylation.

In this study, circular dichroism (CD) was adopted to clarify the mechanisms for the inhibition and acceleration of insulin proteolysis by acylation, since CD revealed the association characteristics of insulin and its acyl derivatives.

Table IV. Minimum Molar Ellipticities of the CD Spectra of Insulin and Its Acyl Derivatives at about 270 nm

	$[\theta]_{\lambda\,\text{minimum}}$ (deg · cm^2/dmol) × 10^{-4}
Native Insulin	−1.48
Cap-1	−1.05
Cap-2	−1.12
Lau-1	−0.90
Lau-2	−0.32
Pal-1	−0.73
Pal-2	0.18

The antiparallel beta-structure formed by insulin association exhibits optical activity. The negative CD absorption at 270 nm represents the aromatic residues of phenylalanine and tyrosine in the $B_{23\text{-}28}$ region of the antiparallel beta-structure. Therefore, the attenuation of negative maxima at about 270 nm describes the dissociation of insulin aggregates (19,20). The CD spectra of monoacyl derivatives at about 270 nm were slightly attenuated with an increase in the carbon numbers of the fatty acid, whereas the CD spectra of diacyl derivatives were remarkably attenuated. Therefore, insulin association is inhibited by acylation, especially the diacylation of B_1-phenylalanine and B_{29}-lysine. The first step of insulin association is the formation of a dimer by the hydrophobic interaction of aromatic residues present in $B_{23\text{-}28}$. Furthermore, the N-terminal side of the B chain of insulin takes part in the subsequent formation of tetramers and hexamers (21). Therefore, we speculate that the steric hindrance of the fatty acid attached to lysine-B_{29} inhibits the association of diacyl monomers into dimers, whereas the fatty acid attached to phenylalanine-B_1 of monoacyl insulin sterically hinders the association of dimers into tetramers and hexam-

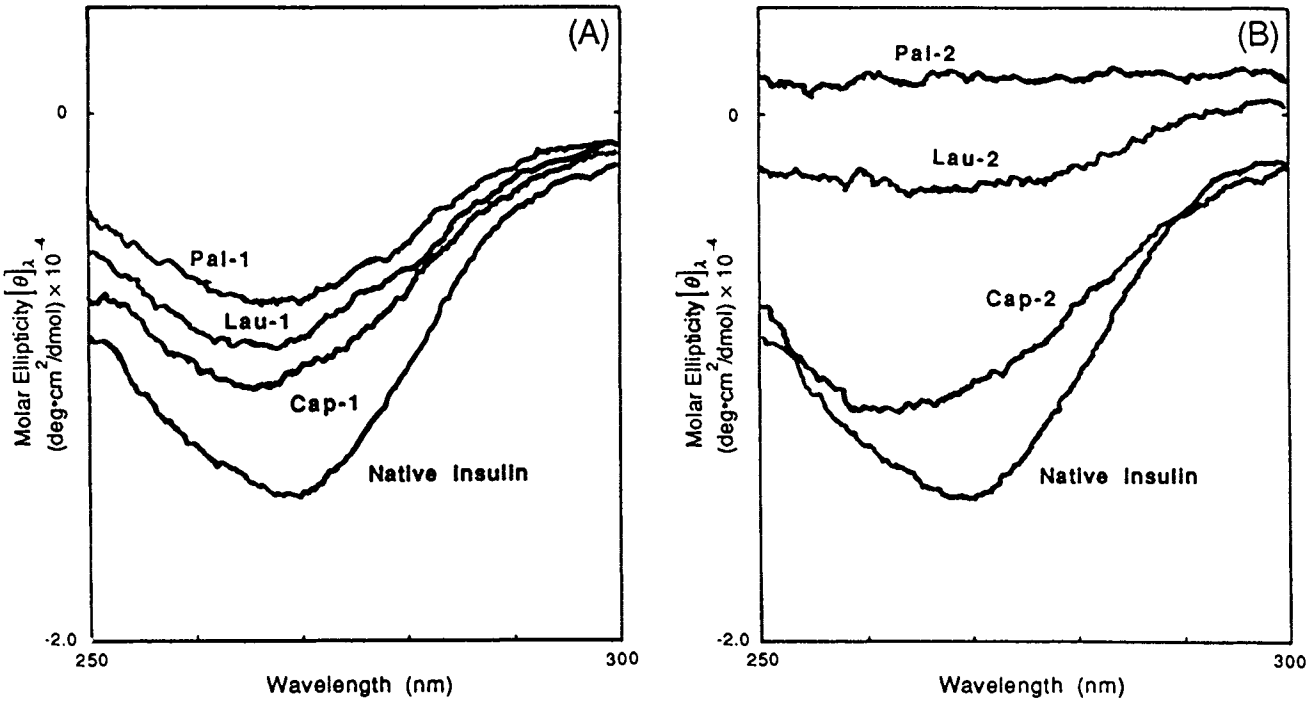

Fig. 3. The circular dichroic spectra of 0.05 mM (A) insulin and monoacyl derivatives and (B) insulin and diacyl derivatives.

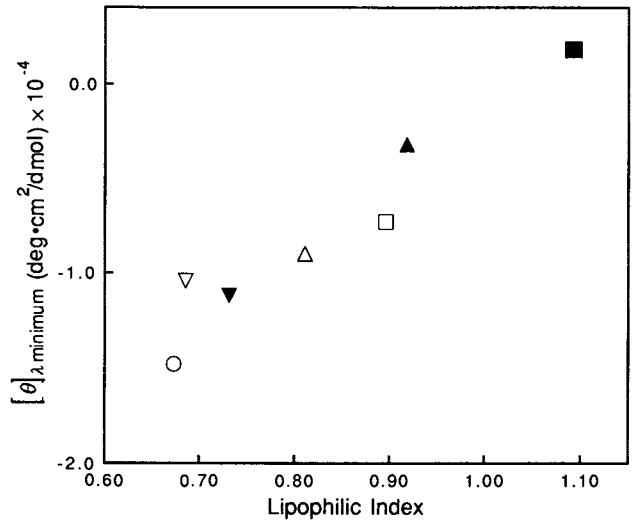

Fig. 4. The relationship between the minimum molar ellipticities at about 270 nm of the circular dichroic spectra of insulin and its acyl derivatives and lipophilicity. Key: (○), Native insulin; (▽), Cap-1; (▼), Cap-2; (△), Lau-1; (▲), Lau-2; (□), Pal-1; (■), Pal-2.

ers. An increase in the monomers available for insulin proteolysis contributes to instability of the diacyl derivatives. On the other hand, monoacyl derivatives were more stable than native insulin despite some dissociation. This result may be explained by the balance between protection of proteolysis by steric hindrance and the acceleration of proteolysis by dissociation.

Our pilot studies demonstrated that caproyl derivatives exhibited adequate pharmacological activities, but activity was reduced with an increase in the carbon number of the fatty acid attached to insulin. In our previous report, the absorption of palmitoyl insulin from the large intestine was higher than that of native insulin. Such higher absorption characteristics of acyl peptides were also seen in the case of TRH (11) and tetragastrin (9).

A relationship between insulin association and its absorption has been reported. Insulin dissociated by ethylenediaminetetraacetic acid or sodium glycocholate is more susceptible to hydrolysis, and sodium dodecyl sulfate enhances insulin absorption via the distal jejunum/proximal ileum segment because it dissociates insulin (22–24). Furthermore, a mutant Asp-B_{28} analogue with lower association than insulin, was more rapidly absorbed after subcutaneous administration (25). Thus, the inhibition of insulin association by acylation influences absorption and stability.

Aminopeptidase, trypsin and chymotrypsin may be responsible for the hydrolysis of insulin, since bacitracin (an aminopeptidase and glutathione-insulin transhydrogenase inhibitor) and aprotinin and soybean trypsin inhibitor (trypsin and chymotrypsin inhibitors) reduced the level of insulin degradation (26,27,17). Furthermore, the different rates of proteolysis of insulin and its acyl derivatives may be related to regional difference in these enzyme activities in the various intestinal regions. Aminopeptidase activity differs at each site of the small intestine (28). Endopeptidase activity in the large intestine was considerably lower than that in the small intestine of the guinea pig (29).

In conclusion, the chemical modification of insulin with monoacylation can improve insulin stability in the small intestinal fluid and the intestinal mucosa and may enhance its gastrointestinal absorption.

ACKNOWLEDGMENTS

Part of this work was supported by a Grant-in-Aid for Science Research from the Uehara Memorial Foundation, Japan.

REFERENCES

1. A. Ginsburg and H. K. Schachman. Studies on the enzymatic breakdown of proteins. I. Action of chymotrypsin on insulin. *J. Biol. Chem.* 235:108-114 (1960).
2. R. J. Schilling and A. K. Mitra. Degradation of insulin by trypsin and alpha-chymotrypsin. *Pharm. Res.* 8:721–727 (1991).
3. V. H. L. Lee, and A. Yamamoto. Penetration and enzymatic barriers to peptide and protein absorption. *Adv. Drug Delivery Rev.* 4:171–207 (1990).
4. M. Kidron, H. Bar-On, E. M. Berry and E. Ziv. The absorption of insulin from various regions of the rat intestine. *Life Sci.* 31:2837–2841 (1982).
5. S. Fujii, T. Yokoyama, K. Ikegaya, F. Sato and N. Yokoo. Promoting effect of the new chymotrypsin inhibitor FK-448 on the intestinal absorption of insulin in rats and dogs. *J. Pharm. Pharmacol.* 37:545–549 (1985).
6. C. Weingarten, A. Moufti, J. Delattre, F. Puisieux and P. Couvreur. Protection of insulin from enzymatic degradation by its association to liposomes. *Int. J. Pharm.* 26:251–257 (1985).
7. M. Shichiri, Y. Shimizu, Y. Yoshida, R. Kawamori, M. Fukuchi, Y. Shigeta and H. Abe. Enteral absorption of water-in-oil-in water insulin emulsions in rabbits. *Diabetologia* 10:317–321 (1974).
8. S. Muranishi, A. Sakai, K. Yamada, M. Murakami, K. Takada and Y. Kiso. Lipophilic peptides: Synthesis of lauroyl thyrotropin-releasing hormone and its biological activity. *Pharm. Res.* 8:649–652 (1991).
9. T. Tenma, E. Yodoya, S. Tashima, T. Fujita, M. Murakami, A. Yamamoto and S. Muranishi. Development of new lipophilic derivatives of tetragastrin: Physicochemical characteristics and intestinal absorption of acyltetragastrin derivatives in rats. *Pharm. Res.* 10:1488–1492 (1993).
10. M. Hashimoto, K. Takada, Y. Kiso and S. Muranishi. Synthesis of palmitoyl derivatives of insulin and their biological activities. *Pharm. Res.* 6:171–176 (1989).
11. K. Yamada, M. Murakami, A. Yamamoto, K. Takada and S. Muranishi. Improvement of intestinal absorption of thyrotropin-releasing hormone by chemical modification with lauric acid. *J. Pharm. Pharmacol.* 44:717–721 (1992).
12. M. Hashizume, T. Douen, M. Murakami, A. Yamamoto, K. Takada and S. Muranishi. Improvement of large intestinal absorption of insulin by chemical modification with palmitic acid in rats. *J. Pharm. Pharmacol.* 44:555–559 (1992).
13. D. G. Lindsay and S. Shall. Acetoacetylation of insulin. *Biochem. J.* 115:587–595 (1969).
14. H. Zahn, D. Brandenburg and H.-G. Gattner. Molecular basis of insulin action: Contributions of chemical modifications and synthetic approaches. *Diabetes* 21:468–475 (1972).
15. K. Takada and Y. Ushirogawa. Effect of pH, dietary proteins and trypsin inhibitors on the hydrolytic rate of human granulocyte colony-stimulating factor (G-CSF) by rat digestive enzymes. *J. Pharmacobio-Dyn.* 14:363–370 (1991).
16. A. Yamamoto, E. Hayakawa and V. H. L. Lee. Insulin and proinsulin proteolysis in mucosal homogenates of the albino rabbit: Implications in peptide delivery from nonoral routes. *Life Sci.* 47:2465–2474 (1990).
17. M. Morishita, I. Morishita, K. Takayama, Y. Machida and T. Nagai. Novel oral microspheres of insulin with protease inhibitor protecting from enzymatic degradation. *Int. J. Pharm.* 78:1–7 (1992).

18. I. Morishita, M. Morishita, K. Takayama, Y. Machida and T. Nagai. Hypoglycemic effect of novel oral microspheres of insulin with protease inhibitor in normal and diabetic rats. *Int. J. Pharm.* 78:9–16 (1992).
19. M. J. Ettinger and S. N. Timasheff. Optical activity of insulin. I. On the nature of the circular dichroism bands. *Biochemistry* 10:824–831 (1971).
20. J. Goldman and F. H. Carpenter. Zinc binding, circular dichroism, and equilibrium sedimentation studies on insulin (bovine) and several of its derivatives. *Biochemistry* 13:4566–4574 (1974).
21. T. L. Blundell, J. F. Cutfield, S. M. Cutfield, E. J. Dodson, G. G. Dodson, D. C. Hodgkin and D. A. Mercola. Three-dimensional atomic structure of insulin and its relationship to activity. *Diabetes* 21:492–505 (1972).
22. F-y. Liu, D. O. Kildsig and A. K. Mitra. Insulin aggregation in aqueous media and its effect on alpha-chymotrypsin-mediated proteolytic degradation. *Pharm. Res.* 8:925–929 (1991).
23. Y. Li, Z. Shao and A. K. Mitra. Dissociation of insulin oligomers by bile salt micelles and its effect on α-chymotrypsin-mediated proteolytic degradation. *Pharm. Res.* 9:864–869 (1992).
24. Z. Shao, Y. Li, R. Krishnamoorthy, T. Chermak and A. K. Mitra. Differential effects of anionic, cationic, nonionic, and physiologic surfactants on the dissociation, α-chymotryptic degradation, and enteral absorption of insulin hexamers. *Pharm. Res.* 10:243–251 (1993).
25. J. Brange, U. Ribel, J. F. Hansen, G. Dodson, M. T. Hansen, S. Havelund, S. G. Melberg, F. Norris, K. Norris, L. Snel, A. R. Sørensen and H. O. Voigt. Monomeric insulins obtained by protein engineering and their medical implications. *Nature* 333:679–682 (1988).
26. E. L. Smith, R. L. Hill and A. Borman. Activity of insulin degraded by leucine aminopeptidase. *Biochim. Biophys. Acta* 29:207–208 (1958).
27. R. A. Roth. Bacitracin: An inhibitor of the insulin degrading activity of glutathione-insulin transhydrogenase. *Biochem. Biophys. Res. Commun.* 98:431–438 (1981).
28. R. E. Stratford, Jr. and V. H. L. Lee. Aminopeptidase activity in homogenates of various absorptive mucosae in the albino rabbit: Implications in peptide delivery. *Int. J. Pharm.* 30:73–82 (1986).
29. P. Kopečková, K. Ikesue, L. Fornůsek and J. Kopeček. Cleavage of peptide bonds by guinea pig brush border membrane enzymes. *Proc. Int. Symp. Control. Rel. Bioact. Mat.* 17:130–131 (1990).